(51) International Patent Classification⁷: A61L 2/00

(21) International Application Number: PCT/US01/02555

(22) International Filing Date: 25 January 2001 (25.01.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/494,814   31 January 2000 (31.01.2000)   US
09/699,284   26 October 2000 (26.10.2000)   US

(71) Applicant (for all designated States except US): THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 1111 Franklin Street, 12th Floor, Oakland, CA 94607-5200 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): PRUSINER, Stanley, B. [US/US]; 400 Pacheco Street, San Francisco, CA

94116 (US). SUPATTAPONE, Surachai [US/US]; 225 Buckingham Way #702, San Francisco, CA 94132 (US). SCOTT, Michael, R. [GB/US]; 1200 Clayton Street #9, San Francisco, CA 94114 (US).

(74) Agent: BOZICEVIC, Karl; Bozicevic, Field & Francis LLP, Suite 200, 200 Middlefield Road, Menlo Park, CA 94025 (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: COMPOSITIONS TREATED TO INACTIVATE INFECTIOUS PROTEINS
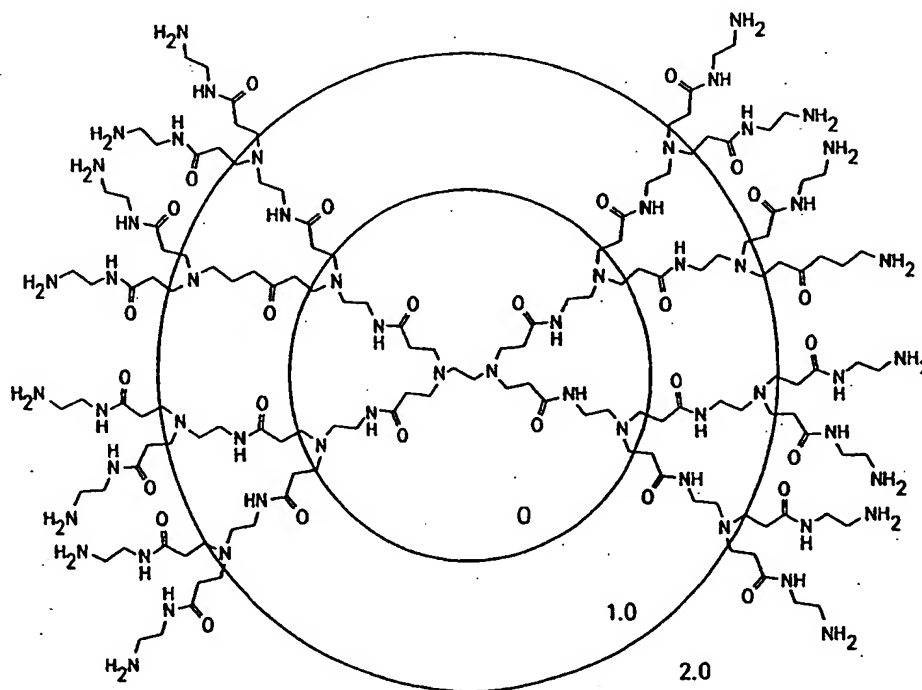
(57) Abstract: An antiseptic composition useful in destroying the infectivity of infectious proteins such as prions is disclosed. The antiseptic composition is preferably maintained at a pH of 4.0 or less which allows for an environment under which the active component destroys infectivity. Methods of disinfecting medical instruments, cell culture extracts, gelatin for capsules and other material are also disclosed.

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COMPOSITIONS TREATED TO INACTIVATE INFECTIOUS PROTEINS

FIELD OF THE INVENTION

5   The present invention relates generally to compositions which have been treated to inactivate infectious proteins such as infectious prions.

BACKGROUND OF THE INVENTION

Antiseptic compositions have been known for over 100 years. In addition to various

10   compositions there are a range of different methods which are known to be effective in killing bacteria and inactivating viruses. Such methods include the use of high temperature alone or in combination with radiation over sufficient periods of time to kill bacteria or disrupt viruses and thereby inactivate them.

Examples of fast acting topical antiseptic compositions are disclosed within U.S. Patent

15   6,110,908 issued August 29, 2000. Another antibacterial composition is disclosed within U.S. Patent 6,025,312 issued February 15, 2000. Examples of other antiseptic compositions are taught within U.S. Patents 5,336,432 issued August 9, 1994; 5,308,611 issued May 3, 1994; 6,106,773 issued August 22, 2000 and 6,096,216 issued August 1, 2000.

Conventional antiseptic compositions and antiseptic methodologies are generally insufficient

20   for inactivating infectious proteins such as prions. Although prions can be inactivated by relatively high temperatures over very long periods of time the temperature ranges and time periods generally used to kill bacteria and inactivate the viruses are insufficient to inactivate prions. One approach to solving this problem is to attempt to remove prions from solutions and a chromographic removal process is disclosed within U.S. Patent 5,808,011. Further, others have attempted to provide

25   compositions and methodologies which are intended to inactivate prions as is taught within U.S. Patent 5,633,349 issued May 27, 1997. However, such processes generally take relatively long periods of time e.g. more than 12 hours and generally do not provide a solution which could be readily and economically utilized in order to inactivate prions on a wide range of surfaces such as medical devices.

The present invention offers an antiseptic composition and method for inactivating prions as

30   described further below.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a schematic drawing of a dendrimer molecule showing the defined "generations" of homodisperse structure created using a repetitive divergent growth technique. The specific diagram is

35   of PAMAM, generation 2.0 (ethylene diamine core).

Figure 2 includes 2A and 2B photographs of gels and 2C which is data plotted indicating survival rates. In Figure ⬤ column 3 shows results where no dendrimer ⬤ e added and prions remain whereas dendrimers were added in column 4 showing the removal of PrP^{Sc} present in a cell culture. Figure 2B shows that the use of dendrimers in compositions of the invention does not effect

5   overall protein expression. Figure 2C shows that when cells are assayed for prion infectivity by injection into mice showing the use of dendrimers cures cells.

Figure 3 includes gel panels 3A and 3B and within Figure 3A columns 3 and 4 labeled +PK (proteinase K) show that dendrimers are effective in removing prions best at a pH of less than 4 and Figure 3B shows that several different types of dendrimers are effective in inactivating prion

10   infectivity.

Figure 4 includes double gel panel 4A and double gel panel 4B. Within the upper panel 4A there is a showing that different strains of prions have different susceptibility to dendrimers indicating that specific dendrimers could be used to determine the type of infectivity (prion strain) in a sample with similar results shown in Figure 4A.

15   Figure 5 includes gel panels A, B, C and D. In Figure 5 there is a showing that the addition of urea enhances the ability of dendrimers to denature and remove prion infectivity. Figure 5B indicates that the particular dendrimers tested are most effective at approximately 37°C. Figures 5C and 5D show that dendrimer induced inactivation of prions is irreversible.

Figure 6 includes high resolution photographs 6A and 6B which provide a visualization of

20   what happens to prion rods exposed to dendrimers.

Figure 7 includes photographs of 7A, 7B and 7C using a fluorescein-labeled PPI which demonstrates that dendrimers are effective inside lysosomes.

Figure 8 includes gel panels 8A (2 hours) and 8B (5 minutes). Within Figure 8A column 4 (+) shows that SDS at a pH of about 3.3 completely eliminates PrP^C whereas 1% SDS at a pH of 7.0 is

25   not effective. Figure 8B within column 4 shows that 1% SDS at a pH of 3.3 is effective in inactivating prions after only five minutes of exposure.

Figure 9 includes a gel panel wherein columns show results carried out at different temperatures indicating that the optimal temperature for using acetic SDS to eliminate prions is greater than 20°C.

30   Figure 10 shows that urea (column 4) is also effective in inactivating prions under acetic conditions.

Figure 11 shows four separate gels with each of the gels run at nine different specific pH levels showing that SDS does denature PrP^{Sc} at low pH and high pH but not at a relatively neutral pH and further showing that increasing the percent concentration of SDS improves the ability of the

35   formulation to denature PrP^{Sc}.

Figure 12 provides gel panels which show that acetic buffers other than acetic acid and sodium acetate can be used in combination with SDS in order to denature PrP^{Sc}.

SUMMARY OF THE INVENTION

5   An antiseptic composition is disclosed which is comprised of a first agent which maintains the pH of the composition at about 5.0 or less preferably 4.0 or less; and a second agent characterized by its ability to destroy prion infectivity in the low pH environment created by the first component. The first agent may be any well known acid present in an aqueous solution at sufficient molarity so as to reduce the pH of the antiseptic composition to 5.0 or less and preferably 4.0 or less and maintain the

10   pH at that low level when the antiseptic composition is applied to an object or mixed with a material to be treated. The second agent or active agent is characterized by its ability to inactivate prions (destroy infectivity) when held in the acid environment for as little as two hours or less. The composition of the invention will vary due to the large number of different acids and inactivating agents that can be used. As temperature is increased and pH is lowered the inactivation occurs more rapidly. Compositions of

15   the invention preferably inactivate prions in about 2 hours or less at a pH of about 4.0 or less at temperatures of about 15°C to 40°C. However, low temperatures (e.g. 0°C or higher) and high temperatures (e.g. 100°C or less) can be used as can longer time periods. The inactivation can occur more quickly (e.g. in one minute or less) and can be carried out at lower pH levels (e.g. 3.0 or less) and at higher temperatures (e.g. greater than 40°C).

20   The antiseptic composition is preferably combined with an additional component which results in the formation of treated food, pharmaceutical material or an object selected from the group consisting of a pharmaceutical gel capsule, a gel coated tablet, a blood extender or blood replacement solution, a surgical implant, a bandage, a suture, a dental implement, a dental sponge, a surgical sponge, a candy containing a gelatin such as a caramel candy, a marshmallow, or a mint such as an

25   Altoid ® mint, doughnut glaze, fruit juice, wine, beer, sour cream, yogurt, cottage cheese, ice cream, margarine, and chewing gum. In essence, the antiseptic composition can be conbined with or coated onto any material which may include infectious prions and particularly with any material comprising animal products, most particularly bovine products such as bovine gelatin.

The inactivating component or active agent is best described functionally as those skilled in

30   the art reading this disclosure will contemplate other agents which could be used in a low pH composition to inactivate prions when the basic concepts and specific examples of the invention are described. Some general classes of compounds useful as the active agent include protein denaturants, inorganic salts; organic solvents, detergents and dendrimers.

A method is disclosed whereby any type of object can be sterilized by combining normal

35   sterilization procedures with the use of an antiseptic composition of the invention which is capable of

rendering a conformationally altered protein such as a prion non-infectious. The method is particularly useful in sterilizing me⬤ devices such as surgical instruments and cath⬤s which have been used and brought into contact with blood or brain tissue. Objects sterilized via the method are also part of the invention and include capsules which are made from gelatin extracted from cattle which cattle may

5   be infected with prions, i.e. have undiagnosed BSE known as "mad cow disease."

The antiseptic compositions of the invention can be combined with conventional antibacterial and antiviral agents in aqueous or alcohol solutions to produce disinfecting agents or surgical scrubs. Branched polycations for use in the invention include, but are not limited to, polyamidoamide (PAMAM), polypropyleneimine (PPI), and polyethyleneimine (PEI) dendrimers,

10   poly(4'-aza-4'-methylheptamethylene D-glucaramide), polyamidoamines and suitable fragments and/or variants of these compounds. Although polycationic dendrimers can be used as the active agent in antiseptic formulations of the invention there are other more preferred compounds which inactivate prions in an acid environment. The essence of the invention disclosed here is that a wide range of different types of compounds will render prions non-infectious in a relatively short period of time (e.g.

15   2 hours or less) when maintained at a pH of 4.0 or less at moderate temperatures, e.g. 15° to 30° centigrade. In some cases the commercial value of the invention is decreased if the composition does not accomplish its intended purpose in a short time period (e.g. less than 10 minutes at about room temperature 20°C ± 5°C.

An overall aspect of the invention is an antiseptic composition comprising an aqueous solvent,

20   an acid capable of maintaining the composition at a preferred pH of 4.0 or less and an active component which at a low pH renders infectious prions non-infectious.

Another aspect of the invention is the use of a wide range of protein denaturants at low pH to inactivate prions.

An aspect of the invention is a method of treating objects with the antiseptic composition

25   characterized by its ability to render proteins associated with diseases non-infectious.

An advantage of the invention is that proteins such as prions can be rendered non-infectious without the need for extreme conditions such as exposure to heat over long periods of time, e.g. without the need for an exposure of 1-10 hours at 100°-200°C.

A feature of the invention is that compositions can be useful while containing only very low

30   concentrations of the prion inactivating component such as SDS or polycationic dendrimers, e.g. 1% to 0.001%.

Another aspect of the invention is that capsules made with bovine gelatin can be certified prion free.

Another aspect of the invention is that drugs produced from cell cultures treated with an active

35   component such as SDS, or polycationic dendrimers can be certified prion free.

-4-

Still another aspect of the invention is that medical devices being reused after exposure to blood or brain tissue c█████certified prion free. ●

Still another aspect of the invention is that hospitals, operating rooms and devices and equipment within them can be certified prion free by contacting them with polycationic dendrimers at

5   standard temperatures and pressures.

An advantage of the invention is that conformationally altered protein such as prions can be rendered non-infectious with a method which need only consist of applying an active component such as SDS or a polycationic dendrimer preferably held at a pH of 4.5 or less.

Another aspect of the invention is soaps, surgical scrubs, detergents and the like are formulated

10   to contain the acid component and the prion inactivating component.

Other aspects of the invention are methods of sterilizing manufacturing equipment such as chromatographic columns and using the sterilized equipment to manufacture products such as pharmaceuticals.

Another aspect is the use of compositions to sterilize dental instruments, equipment and

15   offices.

An advantage of the invention is that compositions comprised of the inactivating component and acid component can be used to inactivate prions which might be present on surgical instruments, knives and/or other tools or equipment used by butchers, particularly those used in the butchering of cows or other animals which might be infected with prions.

20   A feature of the invention is that compositions of the invention can be effective in activating prions when the inactivating component (e.g. SDS or polycationic dendrimers) are present in very low concentrations, e.g. 1% to 0.001% or less.

An important aspect of the invention is an assay whereby multiple compounds can be quickly and easily tested for their ability to destroy the infectious character of prions while the compound and

25   the prions are held in a low pH environment.

These and other aspects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the compounds, and assay method more fully described below.

30   DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Before the present methods, objects and compositions are described, it is to be understood that this invention is not limited to the particular steps, devices or components described and, as such, may of course vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the

35   present invention will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood ⬤one of ordinary skill in the art to which this i⬤ion belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All

5   publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of

10   publication provided may be different from the actual publication dates which may need to be independently confirmed.

DEFINITIONS

The term "acid" is used to describe any compound or group of compounds which has one or

15   more characteristics of (a) sour taste; (b) turns litmus dye red; (c) reacts with certain metals to form a salt; (d) reacts with certain bases or alkalines to form a salt. An acid comprises hydrogen and in water undergoes ionization so that H_3O^+ ions are formed - also written as H^+ and referred to as hydronium ions or simply hydrogen ions. Weak acids such as acetic acid or carbonic acid may be used as may strong acids such as hydrochloric acid, nitric acid and sulfuric acid (HCl, H_2SO_4, H_2NO_3). In

20   compositions of the invention the acid is preferably present in a concentration so as to obtain a pH of 5 or less, more preferably 4 or less and still more preferably 3.5 ± 1. The acid component of the antiseptic composition must be present in a concentration (molarity) to keep the composition in the desired pH range. The concentration (molarity) or the acid used will vary somewhat with the particular acid used, the solvent used (water or alcohol) and other factors such as temperature and pressure. The

25   acid component is preferably present in sufficient molarity and is of such type that when the antiseptic composition of the invention is put into use (e.g. mixed with a sample to be disinfected) the composition remains within the preferred pH range. Thus, stronger and/or more concentrated forms of acids are preferred when the composition is to be used on or in a situation where the composition will be significantly diluted and/or contact a high pH (i.e. very basic component).

30   The terms "active component," "active agent," "inactivating agent" and the like are used interchangeably herein to describe a compound or group of compounds which when combined in the "acid" component of the invention in the antiseptic composition will render a conformationally altered protein non-infectious. Preferably the active component is within an environment of a pH of about 5 or less, preferably 4 or less and in a low concentration e.g. less than 5% by volume of the composition

35   and with inactive prions or other conformationally altered proteins in two hours or less at about room

-6-

temperature 15°C to 30°C. Active components can be determined using an assay of the invention whereby different com⬤ds are tested for their ability to destroy infecti⬤ Preferred compounds which act as an active component for prions include SDS, urea, and a wide range of protein . denaturants including guanidine and thiocynate as well as various branched polycations. Some non-limiting examples of compounds which could be used as the active component include the following:

1)   conventional protein denaturants including:

    a)   urea
    b)   guanidine
    c)   guanidine hydrochloride
    d)   beta-mercaptoethanol
    e)   dithiothreitol (DTT)
    f)   chaotropes

2)   inorganic salts including:
    a)   lithium bromide
    b)   thiocyanate
    c)   potassium thiocyanate
    d)   sodium iodide
    e)   ammonium chloride, EDTA (metal chelator)
    f)   lithium ion and salts thereof
    g)   formic acid and salts thereof

3)   organic solvents including:
    a)   formamide
    b)   dimethylformamide
    c)   dichloro- and trichloroacetic acids and their salts
    d)   trifluroethanolamine (TFE)

4)   detergents including:
    a)   sodium dodecyl sulfate (SDS) (also known as lauryl sulfate, sodium salt -- other salts are also useful including lithium and potassium salts.
    b)   sodium cholate
    c)   sodium deoxycholate
    d)   octylglucoside
    e)   dodecyldimethylamine oxide
    f)   3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)
    g)   dodecyltriethylammonium bromide (DTAB)
    h)   cetyltrimethylammonium bromide (CTAB)
    i)   polyoxyethylene-p-isooctylphenyl ether (e.g. Triton X-20, Triton X-100, Triton X-114).

5)   Branched Polycations
    a)   polyamidoamide (PAMAM) dendrimers
    b)   polypropyleneimine (PPI) dendrimers
    c)   polyethyleneimine (PEI) dendrimers
    d)   poly (4'-aza-4'-methylheptamethylene D-glycaramide)
    e)   polyamidoamines
    f)   fragments and variants of any of a-e

An aspect of the invention is providing any or all of the compound groups (1-5) above with a material which may contain infectious prions under conditions which inactive the infectivity of the prions which conditions preferably comprise a pH of 4.0 or less, a temperature of 15° C or higher wherein the inactivation taken place in a period of time of about 2 hours or less, preferably 1 hour or
5   less and more preferably 10 minutes or less.

Further information of compounds and conditions effecting protein conformation can be found in Voet et. al., Biochemistry, pgs 180-280 (1990); Scopes, RK., Protein Purification: Principles and Practice,.pgs. 57-71 (1987); and Deutscher, MP., Guide to Protein Purification, pgs. 240-241 (1990)
10   which are incorporated herein by reference to disclose possible active compounds and condition parameters which should be considered in implementing the invention.

The term "detergent" is used to mean any substance that reduces the surface tension of water. Examples of detergents are provided above as possible active components. The detergent may be a surface active agent which concentrates at oil-water interfaces, exerts emulsifying action and thereby
15   aids in removing soils e.g. common sodium soaps of fatty acids. A detergent may be anionic, cationic, or monionic depending on their mode of chemical action. Detergents include linear alkyl sulfonates (LAS) often aided by "builders." A LAS is preferably an alkyl benzene sulfonate ABS which is readily decomposed by microorganisms (biodegradable). The LAS is generally a straight chain alkyl comprising 10 to 30 carbon atoms. The detergent may be in a liquid or a solid form.

20   The terms "prion", "prion protein", "PrP^{Sc} protein" and the like are used interchangeably herein to refer to the infectious PrP^{Sc} form of a PrP protein, and is a contraction of the words "protein" and "infection." Particles are comprised largely, if not exclusively, of PrP^{Sc} molecules encoded by a PrP gene. Prions are distinct from bacteria, viruses and viroids. Known prions infect animals to cause scrapie, a transmissible, degenerative disease of the nervous system of sheep and goats, as well as
25   bovine spongiform encephalopathy (BSE), or "mad cow disease", and feline spongiform encephalopathy of cats. Four prion diseases known to affect humans are (1) kuru, (2) Creutzfeldt-Jakob Disease (CJD), (3) Gerstmann-Sträussler-Scheinker Disease (GSS), and (4) fatal insomnia (FI). As used herein "prion" includes all forms of prions causing all or any of these diseases or others in any animals used - and in particular in humans and domesticated farm animals.
30   The term "conformationally altered protein" is used here to describe any protein which has a three dimensional conformation associated with a disease. The conformationally altered protein may cause the disease, be a factor in a symptom of the disease or appear as a result of other factors. The conformationally altered protein appears in another conformation which has the same amino acid sequence. In general, the conformationally altered protein formed is "constricted" in conformation as
35   compared to the other "relaxed" conformation which is not associated with disease. Those skilled in

the art reading this disclosure will recognize the applicability of the antiseptic composition of the invention to other conf⬤ionally altered proteins even though the inven⬤s described in general as regards to prions. The following is a non-limiting list of diseases with associated proteins which assemble two or more different conformations wherein at least one conformation is an example of a

5   conformationally altered protein.

Disease	Insoluble Proteins
Alzheimer's Disease	APP, Aβ peptide, α1-antichymotrypsin, tau, non-Aβ component, presenillin 1, presenillin 2 apoE
Prion diseases, Creutzfeldt Jakob disease, scrapie and bovine spongiform encephalopathy	PrP^{Sc}
ALS	SOD and neurofilament
Pick's disease	Pick body
Parkinson's disease	α-synuclein in Lewy bodies
Frontotemporal dementia	tau in fibrils
Diabetes Type II	Amylin
Multiple myeloma-- plasma cell dyscrasias	IgGL-chain
Familial amyloidotic polyneuropathy	Transthyretin
Medullary carcinoma of thyroid	Procalcitonin
Chronic renal failure	$β_2$--microglobulin
Congestive heart failure	Atrial natriuretic factor
Senile cardiac and systemic amyloidosis	Transthyretin
Chronic inflammation	Serum amyloid A
Atherosclerosis	ApoA1
Familial amyloidosis	Gelsolin
Huntington's disease	Huntingtin

The terms "sterilizing", "making sterile" and the like are used here to mean rendering something non-infectious or render███someth:ng incapable of causing a disease. Sp███cally, it refers to rendering a protein non-infectious or incapable of causing a disease or the symptoms of a disease. Still more specifically, it refers to rendering a conformationally altered protein (e.g. PrP^{Sc} known as prions)

5   incapable of causing a disease or the symptoms of a disease.

By "effective dose" or "amount effective" is meant an amount of a compound sufficient to provide the desired sterilizing result, *e.g.*, eliminate the infectivity of any prions present. This will vary depending on factors such as (1) the active agent used, (2) the pH of the antiseptic composition, (3) the type of object or material being sterilized, and (4) the amount or concentration of infectious proteins

10   which might be present. Polycations of the invention or more specifically polycationic dendrimer compounds of the invention could be mixed with a material in an amount in a range 1 to 500 μg of dendrimer per ml or mg of material being sterilized. The concentration is sufficient if the resulting composition is effective in decreasing (preferably eliminating) the infectivity of conformationally altered proteins such that the treated material over time would not result in infection. Because (1) some

15   materials will have higher concentrations of altered protein than others (2) some materials are contacted more frequently than others and (3) individual proteins have different degrees of infectivity the effective dose or concentration range needed to sterilize can vary considerably. It is also pointed out that the dose needed to treat an amount of material may vary somewhat based on the pH the treatment is carried out at and the amount of time the compound is maintained in contact with the material at the desired low pH

20   (e.g., 4.5 or less) level and the surrounding temperature and pressure.

The term "LD$_{50}$" as used herein is the dose of an active substance that will result in 50 percent lethality in all treated experimental animals. Although this usually refers to invasive administration, such as oral, parenteral, and the like, it may also apply to toxicity using less invasive methods of administration, such as topical applications of the active substance.

25   The term "amine-terminated" includes primary, secondary and tertiary amines.

The terms "PrP protein", "PrP" and like are used interchangeably herein and shall mean both the infectious particle form PrP^{Sc} known to cause diseases (spongiform encephalopathies) in humans and animals and the noninfectious form PrP^{C} which, under appropriate conditions is converted to the infectious PrP^{Sc} form.

30   The term "PrP gene" is used herein to describe genetic material which expresses proteins including known polymorphisms and pathogenic mutations. The term "PrP gene" refers generally to any gene of any species which encodes any form of a prion protein. Some commonly known PrP sequences are described in Gabriel et al., *Proc. Natl. Acad. Sci. USA* 89:9097-9101 (1992) and U.S. Patent No. 5,565,186, incorporated herein by reference to disclose and describe such sequences. The PrP gene can

35   be from any animal, including the "host" and "test" animals described herein and any and all

-10-

polymorphisms and mutations thereof, it being recognized that the terms include other such PrP genes that are yet to be disco●●. The protein expressed by such a gene can a●●e either a PrP^C (non-disease) or PrP^{Sc} (disease) form.

5 The terms "standardized prion preparation", "prion preparation", "preparation" and the like are used interchangeably herein to describe a composition (e.g., brain homogenate) obtained from the brain tissue of mammals which exhibits signs of prion disease: the mammal may (1) include a transgene as described herein; (2) have and ablated endogenous prion protein gene; (3) have a high number of prion protein gene from a genetically diverse species; and/or (4) be a hybrid with an ablated endogenous prion protein gene and a prion protein gene from a genetically diverse species. Different combinations of 1-4

10 are possible, *e.g.*, 1 and 2. The mammals from which standardized prion preparations are obtained exhibit clinical signs of CNS dysfunction as a result of inoculation with prions and/or due to developing the disease of their genetically modified make up, *e.g.*, high copy number of prion protein genes. Standardized prion preparations and methods of making such are described and disclosed in U.S. Patent 5,908,969 issued June 1, 1999 and U.S. Patent 6,020,537 issued February 1, 2000, both of which are

15 incorporated herein by reference in their entirety to disclose and describe standardized prion preparations.

The term "Alzheimer's disease" (abbreviated herein as "AD") as used herein refers to a condition associated with formation of neuritic plaques comprising amyloid β protein, primarily in the hippocampus and cerebral cortex, as well as impairment in both learning and memory. "AD" as used herein is meant to encompass both AD as well as AD-type pathologies.

20 The term "AD-type pathology" as used herein refers to a combination of CNS alterations including, but not limited to, formation of neuritic plaques containing amyloid β protein in the hippocampus and cerebral cortex. Such AD-type pathologies can include, but are not necessarily limited to, disorders associated with aberrant expression and/or deposition of APP, overexpression of APP, expression of aberrant APP gene products, and other phenomena associated with AD. Exemplary AD-

25 type pathologies include, but are not necessarily limited to, AD-type pathologies associated with Down's syndrome that is associated with overexpression of APP.

The term "phenomenon associated with Alzheimer's disease" as used herein refers to a structural, molecular, or functional event associated with AD, particularly such an event that is readily assessable in an animal model. Such events include, but are not limited to, amyloid deposition, neuropathological

30 developments, learning and memory deficits, and other AD-associated characteristics.

The term "cerebral amyloid angiopathy" (abbreviated herein as CAA) as used herein refers to a condition associated with formation of amyloid deposition within cerebral vessels which can be complicated by cerebral parenchymal hemorrhage. CAA is also associated with increased risk of stroke as well as development of cerebellar and subarachnoid hemorrhages (Vinters (1987) *Stroke* 18:311-324;

35 Haan et al. (1994) *Dementia* 5:210-213; Itoh et al. (1993) *J. Neurol. Sci.* 116:135-414). CAA can also

be associated with dementia prior to onset of hemorrhages. The vascular amyloid deposits associated with CAA can exist in t███sence of AD, but are more frequently associa███vith AD.

The term "phenomenon associated with cerebral amyloid angiopathy" as used herein refers to a molecular, structural, or functional event associated with CAA, particularly such an event that is readily assessable in an animal model. Such events include, but are not limited to, amyloid deposition, cerebral parenchymal hemorrhage, and other CAA-associated characteristics.

The term "β-amyloid deposit" as used herein refers to a deposit in the brain composed of Aβ as well as other substances.

Abbreviations used herein include:

CNS for central nervous system;

BSE for bovine spongiform encephalopathy;

CJD for Creutzfeldt-Jakob Disease;

FFI for fatal familial insomnia;

GSS for Gerstmann-Sträussler-Scheinker Disease;

AD for Alzheimer's disease;

CAA for cerebral amyloid angiopathy;

Hu for human;

HuPrP for human prion protein;

Mo for mouse;

MoPrP for mouse prion protein;

SHa for a Syrian hamster;

SHaPrP for a Syrian hamster prion protein;

PAMAM for polyamidoamide dendrimers

PEI for polyethyleneimine

PK for proteinase K

PPI for polypropyleneimine

PrP^{Sc} for the scrapie isoform of the prion protein;

PrP^{C} for the cellular contained common, normal isoform of the prion protein;

PrP 27-30 or PrP^{Sc} 27-30 for the treatment or protease resistant form of PrP^{Sc};

$MoPrP^{Sc}$ for the scrapie isoform of the mouse prion protein;

N2a for an established neuroblastoma cell line used in the present studies;

ScN2a for a chronically scrapie-infected neuroblastoma cell line;

ALS for amyotrophic lateral sclerosis;

HD for Huntington's disease;

FTD for frontotemporal dementia;

-12-

SDS for sodium dodecyl sulfate;

SOD for sup⬤⬤e dismutase

⬤

GENERAL ASPECTS OF THE INVENTION

5   The invention encompasses a range of antiseptic compositions, methods of rendering
conformationally altered proteins non-infectious and assays for determining compounds which may be
used as active agents. The composition is comprised of an acid component and an active component,
although a single compound could serve the function of both components.. The composition preferably
comprises a solvent carrier component which is generally alcohol or aqueous based. The acid component
10   is characterized by maintaining the pH of the composition at 5.0 or less and preferably at 4.0 or less
when in use. The active component is characterized by rendering infectious proteins non-infectious.
Preferably the active component in the low pH environment of the composition renders infectious proteins
non-infectious in two hours or less at a temperature of 40° centigrade or less.

Suitable acid components include a non-toxic weak acid such as acetic acid having dissolved
15   therein an active component such as a branched polycation. Compositions of the invention may be in the
form of aqueous or alcohol solutions which are comprised of a branched polycation, an antibacterial, an
antifungal and an antiviral compound. The antiseptic compositions are coated on, mixed with, injected
into or otherwise brought into contact with a material to be sterilized. The composition is applied in a
manner so that the branched polycation is maintained at a low pH (e.g. 5 or less and preferably 3.5 ± 1)
20   in an amount of 1 μg or more polycation per ml or mg of material to be sterilized. The composition is
maintained in the desired pH range at normal temperature (e.g., 15°C to 30°C) for a sufficient period of
time (e.g. preferably less than 2 hours) to cause conformationally altered protein present on or in the
material to be destroyed (e.g. hydrolyzed) or rendered non-infective. Preferred compositions of the
invention are useful in cleaning and sterilizing and may be comprised of an active agent such as SDS or
25   polycationic dendrimers, a detergent, and an acid component providing a pH less than 3.5.

DENDRIMER COMPOUNDS WHICH CLEAR PRIONS

Dendrimers are branched compounds also known as "starburst" or "star" polymers due to a
characteristic star-like structure (see Figure 1). Dendrimers of the invention are polymers with structures
30   built from AB_n monomers, with $n \geq 2$, and preferably n=2 or 3. Such dendrimers are highly branched and
have three distinct structural features: 1) a core, 2) multiple peripheral end-groups, and 3) branching
units that link the two. Dendrimers may be cationic (full generation dendrimers) or anionic (half
generation dendrimers). For a review on the general synthesis, physical properties, and applications of
dendrimers, see, e.g., Tomalia et. al, Angew. *Chem. Int. Ed. Engl.*, 29:138-175, (1990); Y. Kim and C.
35   Zimmerman, *Curr Opin Chem Biol*, 2:733-7421 (1997).

In a preferred embodiment, sterilizing compositions of the invention comprise a cationic
dendrimer preferably d⬤ved in a low pH solvent such as acetic acid. E⬤ples of suitable dendrimers
are disclosed in U.S. Pat. Nos. 4,507,466, 4,558,120, 4,568,737, 4,587,329, 4,631,337, 4,694,064,
4,713,975, 4,737,550, 4,871,779, and 4,857,599 to D. A. Tomalia, et al., which are hereby incorporated

5   by reference to disclose and describe such compounds. Dendrimers typically have tertiary amines which
have a pKa of 5.7. The dendrimers can optionally be chemically or heat treated to remove some of the
tertiary amines. Other suitable cations include polypropylene imine, polyethyleneimine (PEI), which has
tertiary amines with a pKa of 5.9, and poly(4'-aza-4'-methylheptamethylene D-glucaramide), which has
tertiary amines with a pKa of 6.0. The cationic dendrimer is preferably dissolved in the low pH solvent

10   such as vinegar in a concentration of 0.0001% or more, preferably 0.01% or more and more preferably
about 1%.

Preferably, the dendrimers for use in the invention are polyamidoamines (hereinafter
"PAMAM"). PAMAM dendrimers are particularly biocompatible, since polyamidoamine groups
resemble peptide bonds of proteins.

15   Dendrimers are prepared in tiers called generations (see generations 0, 1 and 2 in Figure 1) and
therefore have specific molecular weights. The full generation PAMAM dendrimers have amine terminal
groups, and are cationic, whereas the half generation dendrimers are carboxyl terminated. Full
generation PAMAM dendrimers are thus preferred for use in the present invention. PAMAM dendrimers
may be prepared having different molecular weights and have specific values as described in Table 1

20   below for generations 0 through 10.

TABLE A

LIST OF PAMAM DENDRIMERS AND THEIR
MOLECULAR WEIGHTS (Ethylene Diamine core, amine terminated),

GENERATION	TERMINAL GROUPS	MOL. WT. g/mole
0	4	517
1	8	1430
2	216	3256
3	32	6909
4	64	14,215
5	128	28,795
6	256	58,048
7	512	116,493
8	1024	233,383
9	2048	467,162
10	4096	934,720

As shown in Table A, the number of terminal amine groups for PAMAM dendrimers generations
0 through 10 range from 4 to 4,096, with molecular weights of from 517 to 934,720. PAMAM
dendrimers are available commercially from Aldrich or Dendritech. Polyethyleneimine or polypropylene

40   dendrimers or quaternized forms of amine-terminated dendrimers may be prepared as described by

-14-

Tomalia et. al, Angew, *Chem. Int. Ed. Engl.*, 29:138-175 (1990) incorporated by reference to describe and disclose methods ⬤king dendrimers.

STERILIZING COMPOSITIONS

5   Examples provided here show that various active compounds such as SDS or highly-branched polycations, e.g. dendrimer compounds, at a pH of 4.0 or less affect the extent and distribution of PrP^{Sc} protein deposits in scrapie-infected cells. The presence of these active compounds in a low pH environment and at relatively low, non-cytotoxic levels results in a significant reduction in detectable PrP^{Sc} in cells and brain homogenates. Thus, the present invention encompasses compositions for

10   reducing, inhibiting, or otherwise mitigating the degree of infectivity of a protein. A composition of the invention is comprised of any compound capable of destroying conformationally altered proteins when in a low pH environment, (e.g. a detergent such as SDS or a polycationic dendrimer) in solution, suspension or mixture.

15   ## STERILIZING FORMULATIONS.

         Sterilizing compositions of the invention preferably contain the active component in a concentration from 0.0001 to 10% of the formulation. The following methods and excipients are merely exemplary and are in no way limiting.

         In addition to including the active compound in the formulation it is important to maintain that

20   compound in a low pH environment. Any number of known acids or mixtures of acids could be used with the invention. Non-limiting examples of commercially available products which could be supplemented with the cationic compounds are described below. In these formulations the percentage amount of each ingredient can vary. In general a solvent ingredient (e.g. water or alcohol) is present in amounts of 40% to 100%. The other ingredients are present in an amount in a range of 1% to 60% and more generally

25   5% to 20%. In each case the polycationic compounds of the invention are added in amounts of about 0.01% to 5% and preferably 0.1% to 2% and more preferably about 1%. The amount added is an amount needed to obtain the desired effect.

FORMULATION 1	
Component	**wt %**
acid	90 - 99.99
active component	0.01 - 10

FORMULATION 2	
Component	**wt %**
acid	90 - 99.99
protein denaturant	0.01 - 10

FORMULATION 3	
Component	wt %
acid	90 - 99.99
inorganic salt	0.01 - 10

FORMULATION 4	
Component	wt %
acid	90 - 99.99
organic solvent	0.01 - 10

FORMULATION 5	
Component	wt %
acid	90 - 99.99
detergent	0.01 - 10

FORMULATION 6	
Component	wt %
water	10 - 99
acid	1 - 20
active component of any of 1-5	0.01 - 10

FORMULATION 7	
Component	wt %
water	10 - 98
acid	1 - 20
detergent	1 - 20
polycationic dendrimer	0.01 - 5

FORMULATION 8	
Component	wt %
water	10 - 98
acetic acid	1 - 20
linear alkyl sulfonate	1 - 20
polycationic dendrimer	0.01 - 5

FORMULATION 9	
Component	wt %
water	10 - 99
acetic acid	1 - 20
SDS	0.01 - 10

-16-

FORMULATION 10	
Component	wt %
water	1 - 98
alcohol	0 - 98
acid	1 - 20
detergent	1 - 20
polycationic dendrimer	0.1 - 5

FORMULATION 11	
Component	wt %
water	1 - 99
acid	1 - 20
antibacterial	0.1 - 5
detergent	1 - 20
polycationic dendrimer	0.1 - 5

FORMULATION 12	
Component	wt %
water	3 - 98.889
antimicrobial active agent	0.001 - 5
anionic surfactant	1 - 80
proton (H^+) donating agent	0.1 - 12
polycationic dendrimer	0.01 - 5

FORMULATION 13	
Component	wt %
Polycationic Dendrimer	0.5
Ethanol	74.0
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	1.0
Chain silicone	0.5
Triglyceride	0.5
Lactic acid	10.0
Purified water	13.28

-17-

FORMULATION 14	
Component	wt %
Polycationic Dendrimer	1.0
Ethanol	75.0
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	1.0
Cyclic silicone	0.2
Triglyceride	0.3
Acetic Acid	20.0
Purified water	2.28

FORMULATION 15	
Component	wt %
Polycationic Dendrimer	0.25
Ethanol	74.0
Chlorhexedine gluconate	0.75
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	2.0
Chain silicone	0.2
Cyclic silicone	0.2
Triglyceride	0.3
Acetic Acid	20.0
Purified water	2.08

FORMULATION 16	
Component	wt %
Polycationic Dendrimer	0.1
Ethanol	75.0
Chlorhexedine gluconate	0.9
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	1.0
Chain silicone	0.5
Cyclic silicone	0.5
Triglyceride	0.3
Lactic acid	14.0
Purified water	7.98

-18-

FORMULATION 17

Component	wt %
Polycationic Dendrimer	0.01
Ethanol	75.0
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	2.0
Chain silicone	0.99
Cyclic silicone	2.0
Triglyceride	3.0
Lactic acid	9
Purified water	7.78

FORMULATION 18

Component	wt %
Polycationic Dendrimer	1
Ethanol	75.0
Chlorhexedine gluconate	0.2
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	0.8
Chain silicone	0.2
Cyclic silicone	0.2
Triglyceride	.38
Acetic acid	10
Purified water	12

FORMULATION 19

Component	wt %
Polycationic Dendrimer	0.001
Ethanol	75.99
Chlorhexedine gluconate	0.2
CAE	0.02
Glycerine	1.0
Chain silicone	0.2
Triglyceride	0.3
Lactic acid	14
Purified water	8.28

FORMULATION 20	
Component	wt %
Polycationic Dendrimer	1
Ethanol	75.0
Benzalkonium chloride	0.2
CAE	0.02
1, 3-butylene glycol	1.0
Metylphenyl polysiloxane	0.2
Isopropyl myristate (IPM)	0.3
Purified water	22.28

FORMULATION 21	
Component	wt %
water	1 - 98
alcohol	0 - 98
acid	1 - 20
SDS	1 - 20

FORMULATION 22	
Component	wt %
water	1 - 99
acid	1 - 20
antibacterial agent	0.1 - 5
detergent	1 - 20

FORMULATION 23	
Component	wt %
water	3 - 98.889
antimicrobial active agent	0.001 - 5
anionic surfactant	1 - 80
proton (H^+) donating agent	0.1 - 12
SDS	0.01 - 5

FORMULATION 24	
Component	wt %
SDS	0.5
Ethanol	74.0
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	1.0
Chain silicone	0.5
Triglyceride	0.5
Lactic acid	10.0
Purified water	13.28

FORMULATION 25	
Component	wt %
Urea	1.0
Ethanol	75.0
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	1.0
Cyclic silicone	0.2
Triglyceride	0.3
Acetic Acid	20.0
Purified water	2.28

FORMULATION 26	
Component	wt %
Guanidine hydrochloride	0.25
Ethanol	74.0
Chlorhexedine gluconate	0.75
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	2.0
Chain silicone	0.2
Cyclic silicone	0.2
Triglyceride	0.3
Acetic Acid	20.0
Purified water	2.08

-21-

FORMULATION 27	
Component	wt %
Thiocynate	0.1
Ethanol	75.0
Chlorhexedine gluconate	0.9
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	1.0
Chain silicone	0.5
Cyclic silicone	0.5
Triglyceride	0.3
Lactic acid	14.0
Purified water	7.98

FORMULATION 28	
Component	wt %
Sodium deoxycholate	0.01
Ethanol	75.0
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	2.0
Chain silicone	0.99
Cyclic silicone	2.0
Triglyceride	3.0
Lactic acid	9
Purified water	7.78

FORMULATION 29	
Component	wt %
SDS	1
Ethanol	75.0
Chlorhexedine gluconate	0.2
Benzalkonium chloride	0.2
CAE	0.02
Glycerine	0.8
Chain silicone	0.2
Cyclic silicone	0.2
Triglyceride	.38
Acetic acid	10
Purified water	12

FORMULATION 30	
Component	wt %
SDS	0.001
Ethanol	75.99
Chlorhexedine gluconate	0.2
CAE	0.02
Glycerine	1.0
Chain silicone	0.2
Triglyceride	0.3
Lactic acid	14
Purified water	8.28

FORMULATION 31	
Component	wt %
Sodium Acetate pH 4.0 ±	10%
SDS	4%
Water	86%

FORMULATION 32	
Component	wt %
SDS	4%
Peracetic acid	0.1 – 10%
Water	86 - 95.9%

FORMULATION 33	
Component	wt %
SDS	4%
glycerine	10%
water	86%

FORMULATION 34	
Component	wt %
SDS	1 - 20%
Acid pH 4.0 ± 1	1 -20%
water	60 - 98%

FORMULATION 35	
Component	wt %
SDS	1 - 20%
base pH 10.0 ± 1	1 -20%
water	60 - 98%

FORMULATION 36	
Component	wt %
SDS	1
Ethanol	75.0
Benzalkonium chloride	0.2
CAE	0.02
1, 3-butylene glycol	1.0
Metylphenyl polysiloxane	0.2
Isopropyl Myristate (IPM)	0.3
Purified water	22.28

By using the disclosure provided here and other information such as taught in U.S. Patents 5,767,054; 6,007,831; 5,830,488; 5,968,539; 5,416,075; 5,296,158; and patents and publications cited therein those skilled in the art can produce countless other formulations of the invention. Further, the formulations are preferably adjusted to have a pH of less than 4.0 and such formulations can be used as described in such publications and can be packaged in any suitable container or dispenser device, e.g. taught in 5,992,698. The pH can be lowered with any acid, e.g. HCl, H_2SO_4, H_2NO_3 peracetic acid, etc. can be used as the acid component in the formulae provided above.

Example 17 shows that compounds such as SDS are effective in denaturing PrP^{Sc} not only at a low pH (e.g. 5 or less) but are effective at a high pH (e.g. 9 or more) but are generally not effective at a pH of about 7.0 ± 1. The pH of the formulation can be adjusted to obtain desired results in each particular use. For example, a very high or very low pH may be best for inactivating PrP^{Sc}, these extreme pH's may be undesirable in some situations due to their corrosive effects. Thus, a preferred pH is one which inactivates PrP^{Sc} and has the least possible adverse effects for the intended use. In many situations a preferred pH for an SDS formulation is about 4.0 ± 1 or about 10.0 ± 1.

Formulations of the invention used with a cell culture have the advantage that they are non-toxic. For example, parenteral administration of a solution of the formulations of the invention is preferably nontoxic at a dosage of 0.1 mg/mouse, which is an LD_{50} of less than one at 40 mg/Kg. Various nutrient formulations and/or injectable formulations of the type known to those skilled in the art can be used to prepare formulations for treating cell cultures.

Those skilled in the art will understand that in some situations it may be desirable to further reduce the pH environment to obtain the desired results. This can be accomplished by adding any desired acid. If desired, the pH can be raised to a normal level after treatment is complete, i.e. after a sufficient amount of any conformationally altered protein present are destroyed.

Compounds effective in sterilizing compositions containing conformationally altered proteins are determined via a cell culture assay and an organ homogenate assay each of which is described below in detail.

-24-

On reading th●closure and in particular the description prov●ere on particular assays, other assays will occur to those skilled in the art. The basic concept is (1) providing an acid component in an aqueous and/or alcohol carrier, (2) adding the component (not known to be active) and (3)

5   contacting the test composition with a sample known to contain an infectious protein. After allowing time to pass (e.g. 5 minutes to 2 hours) procedures described here are used to (4) determine if the component is "active", i.e. renders the protein non-infectious. Multiple compounds can be added to the composition and tested simultaneously. If no effect is found then all of the compounds are not active. If a sterilizing effect is found the group of compounds tested can be divided in two and retested until an

10   active compound is specifically identified. Dividing the originally tested group in any manner and retesting can be carried out any number of times.

The active component can be checked against one prion strain at a time or against multiple strains simultaneously. Some active components such as SDS will inactivate all known strains of prion while some polycationic dendrimers will inactivate only specific strains. When the active component

15   inactivates all strains it is useful as an antiseptic and/or therapeutic. When it inactivates only a specific strain it can be used to determine the strain of infectious prion in a sample.

Efforts were made to optimize the transfection of ScN2a cells with pSPOX expression plasmids (Scott, M.R., Köhler, R., Foster, D. & Prusiner, S.B. Chimeric prion protein expression in cultured cells and transgenic mice. Protein Sci. 1, 986-997 (1992)). In connection with those effects an evaluation was

20   made of a transfection protocol that used SuperFect reagent (QIAGEN®). It was found that epitope-tagged (MHM2) PrP^{Sc} (Scott, M.R., Köhler, R., Foster, D. & Prusiner, S.B. Chimeric prion protein expression in cultured cells and transgenic mice. Protein Sci. 1, 986-997 (1992)) could not be detected in ScN2a cells following SuperFect-mediated transfection, whereas MHM2 PrP^{Sc} was efficiently formed when a cationic liposome method for DNA delivery was used. Close scrutiny revealed that, prior

25   to protease digestion, SuperFect-transfected samples expressed MHM2 bands, which are not seen in the background pattern of an untransfected sample. The 3F4 monoclonal antibody does not react with MoPrP but does exhibit high background staining on Western blots of mouse ScN2a cells. Increased immunostaining in the 20-30 kDa region was observed compared to the non-transfected sample. These observations led us to conclude that MHM2 PrP was successfully expressed using SuperFect transfection

30   reagent, but that conversion of MHM2 PrP^{C} to protease-resistant MHM2 PrP^{Sc} was inhibited by SuperFect.

To investigate this apparent inhibition, a Western blot was reprobed with RO73 polyclonal antiserum to detect endogenous $MoPrP^{Sc}$, the presence of which is diagnostic for prion infection in ScN2a cells (Butler, D.A., et al. Scrapie-infected murine neuroblastoma cells produce protease-resistant prion

35   proteins. J. Virol. 62, 1558-1564 (1988)). Surprisingly, it was found that the SuperFect-treated ScN2a

cells no longer contained detectable quantities of MoPrP^{Sc} - also confirmed in Western blots. To investigate the mechani⬤ which SuperFect reduced the level of pre-ex⬤ PrP^{Sc} in chronically infected ScN2a cells, measurements were made of endogenous PrP^{Sc} in ScN2a cells exposed to various concentrations of SuperFect in the absence of plasmid DNA. The results showed that treatment with

5   SuperFect (a branched polycation) caused the disappearance of PrP^{Sc} from ScN2a cells in a dose-dependent manner. The concentration of SuperFect required to eliminate >95% of pre-existing PrP^{Sc} with a three hour exposure was found to be about 150 μg/ml. Duration of treatment also influenced the ability of SuperFect to remove PrP^{Sc} from ScN2a cells: exposure to 150 μg/ml SuperFect for 10 min did not affect PrP^{Sc} levels, whereas 7.5 μg/ml SuperFect eliminated all detectable PrP^{Sc} with a

10   t½= 8 h.

SuperFect is a mixture of branched polyamines derived from heat-induced degradation of a PAMAM dendrimer (Tang, M.X., Redemann, C.T. & Szoka, F.C.J. In vitro gene delivery by degraded polyamidoamine dendrimers. Bioconjug. Chem. 7, 703-714 (1996)). Knowing this structure the ability of several other branched and unbranched polymers to eliminate PrP^{Sc} from ScN2a cells (Table 1). The

15   branched polymers investigated include various preparations of PEI, as well as intact PAMAM and PPI dendrimers. Dendrimers are manufactured by a repetitive divergent growth technique, allowing the synthesis of successive, well-defined "generations" of homodisperse structures (Figure 1). The potency of both PAMAM and PPI dendrimers in eliminating PrP^{Sc} from ScN2a cells increased as the generation level increased. The most potent compounds with respect to eliminating PrP^{Sc} were PAMAM generation

20   4.0 and PPI generation 4.0, whereas PAMAM generation 1.0 showed very little ability to eliminate PrP^{Sc} (Table 1). Similarly, a high MW fraction of PEI was more potent than low MW PEI.

From the foregoing data, it is clear that for all three branched polyamines tested, increasing molecular size corresponded to an increased potency for eliminating PrP^{Sc}. To determine whether this trend was directly attributable to increased surface density of amino groups on the larger molecules,

25   PAMAM-OH generation 4.0 was tested. This is a dendrimer that resembles PAMAM generation 4.0 except that hydroxyls replace amino groups on its surface. Unlike PAMAM generation 4.0, PAMAM-OH generation 4.0 did not cause a reduction of PrP^{Sc} levels even at the highest concentration tested (10 mg/ml), establishing that the amino groups are required for the elimination of PrP^{Sc} by PAMAM (Table 1).

30   In an effort to assess the contribution of the branched architecture to the clearing ability of polyamines for PrP^{Sc}, the linear molecules poly-(L)lysine and linear PEI were also tested. Both of these linear compounds were less potent than a preparation of branched PEI with similar average molecular weight (Table 1), establishing that a branched molecular architecture optimizes the ability of polyamines to eliminate PrP^{Sc}, presumably because the branched structures achieve a higher density of surface amino

35   groups.

Kinetics of PrP^{Sc} elimination by polyamines.

The preceding r⬤ demonstrate the potent ability of branched p⬤ines to clear PrP^{Sc} from ScN2a cells within a few hours of treatment. The utility of these compounds to act as therapeutics for treatment of prion disease was tested by determining whether they were cytotoxic for ScN2a cells, using

5 as criteria cell growth, morphology, and viability as measured by trypan blue staining. None of the compounds was cytotoxic to ScN2a cells after exposure for one week at concentrations up to 7.5 μg/ml. To determine whether branched polyamines can cure ScN2a cells of scrapie infection without affecting cell viability, the kinetics of prion clearance was examined in the presence of a non-cytotoxic concentration (7.5 μg/ml) of three different branched polyamines. ScN2a cells were exposed to

10 SuperFect, PEI, or PAMAM generation 4.0 for varying periods of time. The kinetics of PrP^{Sc} elimination were assessed by Western blotting. All three compounds caused a substantial reduction in PrP^{Sc} levels after 8-16 h of treatment, and of the three compounds, PEI appeared to remove PrP^{Sc} most quickly, with a t½= 4 h.

15 Curing neuroblastoma cells of scrapie infection.

The above results show that it is possible to reverse the accumulation of PrP^{Sc} in ScN2a cells under non-cytotoxic conditions. It was also found that extended exposure to even lower levels of the branched polyamines (1.5 μg/ml) was sufficient to eliminate PrP^{Sc}. Based on these findings, this protocol was used to determine whether the severe reduction in PrP^{Sc} levels following exposure to branched

20 polyamines would persist after removal of the compounds. Following the exposure of ScN2a cells to a 1.5 μg/ml SuperFect for 1 week, PrP^{Sc} was reduced to <1% of the baseline level, but then increased back to ~5% of the baseline level after 3 additional weeks in culture in the absence of polyamine. In contrast, following exposure to 1.5 μg/ml of either PEI or PAMAM generation 4.0 for 1 week, PrP^{Sc} was completely eliminated and did not return even after 3 weeks in culture without polyamines. A more

25 intensive course of treatment with 1.8 μg/ml SuperFect for 9 d also cured ScN2a cells of scrapie infection fully, manifested by the absence of PrP^{Sc} 1 month after removal of SuperFect.

Evidence for polyamines acting within an acidic compartment.

The above results showed the potent activity of branched polyamines in rapidly clearing scrapie

30 prions from cultured ScN2a cells. Based on these results the mechanism by which these compounds act was investigated. All of the compounds which effect removal of PrP^{Sc} from ScN2a cells are known to traffic through endosomes (Boussif, O., et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethyleneimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297-7301 (1995); Haensler, J. & Szoka, F.C.J. Polyamidoamine cascade polymers mediate efficient transfection of

35 cells in culture. Bioconjug. Chem. 4, 372-379 (1993)). Since PrP^{C} is converted into PrP^{Sc} in

-27-

caveolae-like domains (CLDs) or rafts (Gorodinsky, A. & Harris, D.A. Glycolipid-anchored proteins in neuroblastoma cells f○●etergent-resistant complexes without caveolin●●ell Biol. 129, 619-627 (1995); Taraboulos, A., et al. Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibits formation of the scrapie isoform. J. Cell Biol. 129, 121-132

5   (1995); Vey, M., et al. Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proc. Natl. Acad. Sci. USA 93, 14945-14949 (1996); Kaneko, K., et al. COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform. Proc. Natl. Acad. Sci. USA 94, 2333-2338 (1997)) and is then internalized through the endocytic pathway (Caughey, B., Raymond, G.J., Ernst, D. & Race, R.E. N-

10   terminal truncation of the scrapie-associated form of PrP by lysosomal protease(s): implications regarding the site of conversion of PrP to the protease-resistant state. J. Virol. 65, 6597-6603 (1991); Borchelt, D.R., Taraboulos, A. & Prusiner, S.B. Evidence for synthesis of scrapie prion proteins in the endocytic pathway. J. Biol. Chem. 267, 16188-16199 (1992)), it was deduced that polyamines act upon PrP^{Sc} in endosomes or lysosomes. This deduction was investigated by determining the effect of

15   pretreatment with the lysosomotropic agents chloroquine and NH_4Cl on the ability of polyamines to eliminate PrP^{Sc}. These lysosomotropic agents alkalinize endosomes and have no effect on PrP^{Sc} levels when administered to ScN2a cells (Taraboulos, A., Raeber, A.J., Borchelt, D.R., Serban, D. & Prusiner, S.B. Synthesis and trafficking of prion proteins in cultured cells. Mol. Biol. Cell 3, 851-863 (1992)). Experimental results obtained shows that 100 μM chloroquine, but not 30 μM NH_4Cl, blocked the ability

20   of PEI to eliminate PrP^{Sc}. Similar results were obtained with SuperFect and PAMAM, generation 4.0. Although the failure of NH_4Cl to affect PrP^{Sc} levels is not easily explained, the ability of chloroquine to attenuate the ability of branched polyamines to remove PrP^{Sc} is consistent with the notion that these agents act in endosomes or lysosomes.

25   ORGAN HOMOGENATE ASSAY

        The above results with cell cultures prompted investigating the possibility that in an acidic environment branched polyamines, either by indirectly interacting with PrP^{Sc} or with another cellular component, could cause PrP^{Sc} to become susceptible to hydrolases present in the endosome/lysozome. An in vitro degradation assay was developed to evaluate the effect of pH on the ability of polyamines to

30   render PrP^{Sc} sensitive to protease. Crude homogenates of scrapie-infected mouse brain were exposed to a broad range of pH values in the presence or absence of SuperFect and then treated with proteinase K prior to Western blotting. Whereas PrP^{Sc} remained resistant to protease hydrolysis throughout the pH range (3.6-9.6) in the absence of Superfect, addition of the branched polyamine at pH 4.0 or below caused PrP^{Sc} to become almost completely degraded by protease.

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35

Polyamine addition showed a dramatic effect on clearance in vitro which was optimized at pH 4 or less. These results sh⬤at polyamines act on PrP^{Sc} in an acidic compa⬤nt. To establish that the in vitro degradation assay is a valid approximation of the mechanism by which branched polyamines enhance the clearance of PrP^{Sc} from cultured cells, a structure activity analysis was performed with

5   several of the compounds tested in culture cells. An excellent correlation was found between the clearance of PrP^{Sc} in cultured ScN2a cells (Table 1) and the ability to render PrP^{Sc} susceptible to protease at acidic pH in vitro. Notably, PAMAM-OH generation 4.0 failed to render PrP^{Sc} susceptible to protease, whereas PAMAM generation 4.0 and PPI, generation 4.0 exhibited an even stronger activity than Superfect in vitro, as expected from their observed potency in cultured ScN2a cells (Table 1).

10

MECHANISM OF ACTION

When a pH of 4.0 or less was maintained the results show that certain branched polyamines cause the rapid elimination of PrP^{Sc} from ScN2a cells in a dose- and time-dependent manner. These compounds demonstrate a potent ability to remove prions from cultured cells at concentrations that are

15   completely non-cytotoxic. The cells may be maintained indefinitely in culture in the presence of therapeutic levels of branched polyamines. Furthermore, when ScN2a cells were exposed to these compounds for ~ 1 week, PrP^{Sc} was reduced to undetectable levels and remained so for at least one month after removal of the polyamine.

Clarification of the exact mechanism of PrP^{Sc} elimination by branched polyamines is an important

20   objective. Although a number of possible scenarios exist, several possibilities may be excluded already. One possibility that was eliminated was that polyamines act by induction of chaperones such as heat shock proteins that mediate prion protein refolding because the above results show that it was possible to reproduce the phenomenon in vitro. Furthermore polyamines seem to offer advantages over other putative therapeutics that would seek to promote refolding: at very high concentrations, dimethyl

25   sulfoxide (DMSO) and glycerol act as direct "chemical chaperones" and inhibit the formation of new PrP^{Sc} (Tatzelt, J., Prusiner, S.B. & Welch, W.J. Chemical chaperones interfere with the formation of scrapie prion protein. EMBO J. 15, 6363-6373 (1996)); but these compounds cannot reduce pre-existing PrP^{Sc} levels. Furthermore, polyamines inhibit PrP^{Sc} formation at much lower concentrations than these agents. The ability of polyamines to effect the rapid clearance of PrP^{Sc} also contrasts with the activity of

30   other potential prion therapeutics. Sulfated polyanions may inhibit PrP^{Sc} accumulation in ScN2a cells by directly binding to PrP^{C} (Gabizon, R., Meiner, Z., Halimi, M. & Ben-Sasson, S.A. Heparin-like molecules bind differentially to prion-proteins and change their intracellular metabolic fate. J. Cell. Physiol. 157, (1993); Caughey, B., Brown, K., Raymond, G.J., Katzenstein, G.E. & Thresher, W. Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and Congo

red. J. Virol. 68, 2135-2141 (1994)), but because branched polyamines are able to clear pre-existing PrP^{Sc}, their mechanis⬤action cannot simply involve binding to PrP^{C}⬤inhibiting de novo synthesis.

Another possible mechanism which can be excluded is endosomal rupture. The branched polyamines which were effective in clearing PrP^{Sc} from ScN2a cells in our experiments, PEI, SuperFect

5 and PAMAM, are also potent lysosomotropic, osmotic agents which can swell in acidic environments and rupture endosomes (Boussif, O., et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethyleneimine. Proc. Natl. Acad. Sci. U.S.A. 92, 7297-7301 (1995); Haensler, J. & Szoka, F.C.J. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. Bioconjug. Chem. 4, 372-379 (1993)). This might suggest that branched polyamines clear PrP^{Sc}

10 from ScN2a cells by rupturing endosomes and exposing PrP^{Sc} to cytosolic degradation processes. However, it is known that the lysosomotropic, endosome-rupturing agents NH$_4$Cl, chloroquine, and monensin do not interfere with the formation of PrP^{Sc} in ScN2a cells (Taraboulos, A., Raeber, A.J., Borchelt, D.R., Serban, D. & Prusiner, S.B. Synthesis and trafficking of prion proteins in cultured cells. Mol. Biol. Cell 3, 851-863 (1992)). Furthermore, the results also show that chloroquine interferes with

15 the ability of branched polyamines to clear PrP^{Sc} and that polyamines can clear PrP^{Sc} in vitro at acidic pH in the absence of cell membranes. Together, these observations rule out endosome rupture as the mechanism by which branched polyamines remove PrP^{Sc}.

Without committing to any particular mechanism of action it appears likely that branched polyamines require the acidic environment of intact endosomes or lyzosomes to destroy PrP^{Sc}. The

20 structure-activity profile of polymers tested reveals that the most active compounds possess densely packed, regularly-spaced amino groups, suggesting that these compounds may bind to a ligand which has periodically-spaced negative charges. Several scenarios remain possible. (1) Branched polyamines may bind directly to PrP^{Sc} arranged as an amyloid with exposed negatively-charged moieties and induce a conformational change under acidic conditions. (2) Treatment of PrP 27-30 with acid decreases

25 turbidity and increases a-helical content, suggesting that such conditions might dissociate PrP^{Sc} into monomers (Safar, J., Roller, P.P., Gajdusek, D.C. & Gibbs, C.J., Jr. Scrapie amyloid (prion) protein has the conformational characteristics of an aggregated molten globule folding intermediate). It is therefore possible that polyamines bind to an equilibrium unfolding intermediate of PrP^{Sc} present under acidic conditions. (3) Alternatively, polyamines might sequester a cryptic, negatively charged component bound

30 to PrP^{Sc} that is essential for protease resistance, but which is only released when PrP^{Sc} undergoes an acid-induced conformational change. Such a component might act as a chaperone for PrP^{Sc} inside endosomes or lysosomes. (4) Finally, another possibility is that polyamines activate an endosomal or lysosomal factor which can induce a conformational change in PrP^{Sc}. Clearly, more work will be required to determine the precise mechanism by which branched polyamines destroy PrP^{Sc}.

35

GENERAL APPLICABILITY OF ASSAY

The in vitro assay described here is generally applicable in the search for compounds that effectively clear conformationally altered proteins present in food thereby preventing a number of degenerative diseases, where the accumulation of proteins seems to mediate the pathogenesis of these

5 illnesses. By simulating lysosomes, where proteases hydrolyze proteins under acidic conditions, the in vitro brain homogenate assay is able to rapidly evaluate the efficacy of a variety of polyamines to induce degradation of PrP^{Sc}.

The in vitro assay which used scrapie infected brain homogenate to test for compounds which clear PrP^{Sc} could be modified to assay for compounds which would clear any conformationally altered protein.

10 The assay is carried out by homogenizing the organ or tissue where the conformationally altered protein is present in the highest concentration. The pH of the homogenate is then reduced to less than 5.0 and preferably 4.0 or less. For example pancreatic tissue can be homogenized to produce an assay to test for compounds which clear amylin which is associated with type II Diabetes. Homogenized kidney could be used to test for compounds which clear β_2 - microglobulin and homogenized heart or vascular tissue used

15 to test for compounds which clear atrial natriuretic factor. Those skilled in the art will recognize other organs and tissue types which can be homogenized to test for other compounds which clear other conformationally altered proteins.

Besides using the in vitro assay to screen for potential drugs, the compounds found via the assay such as branched polyamines provide a new tool for exploring the conversion of a protein to

20 conformationally altered protein, e.g. PrP^C into PrP^{Sc}. The mechanism by which branched polyamines render PrP^{Sc} susceptible to proteolysis, remains to be established. Whether the interaction of branched polyamines with PrP^{Sc} is reversible is unknown. In addition, we do not know whether branched polyamines are able to solubilize PrP^{Sc} without irreversibly denaturing the protein. Whatever the mechanism by which branched polyamines interact with PrP^{Sc}, it is likely to be different from that found

25 with chaotropes as well as denaturing detergents and solvents (Prusiner, S.B., Groth, D., Serban, A., Stahl, N. & Gabizon, R. Attempts to restore scrapie prion infectivity after exposure to protein denaturants. Proc. Natl. Acad. Sci. USA 90, 2793-2797 (1993))

Using the assays described and disclosed here certain specific branched polyamines have been found which mediate the clearance of PrP^{Sc} from cultured cells under non-cytotoxic conditions. These

30 compounds offer the intriguing possibility of being added to a wide range of low pH food products to neutralize conformational altered proteins present. Since the compounds found act by stimulating normal cellular pathways of protein degradation to destroy PrP^{Sc}, this class of compounds would also likely be of value in the treatment of other degenerative and hereditary disorders where abnormally folded, wild-type or mutant proteins accumulate. Such an approach may find merit in developing an effective therapeutics

for one or more of the common, degenerative illnesses including Alzheimer's disease, Parkinson's disease, amyotrophic █████al sclerosis, frontotemporal dementia, adult on██████diabetes mellitus and the amyloidoses (Beyreuther, K. & Masters, C.L. Serpents on the road to dementia and death. Accumulating evidence from several studies points to the normal function of presenilin 1 and suggests how the mutant

5   protein contributes to deposition of amyloid plaques in Alzheimer's disease. Nature Medicine 3, 723-725 (1997); Masters, C.L. & Beyreuther, K. Alzheimer's disease. BMJ 316, 446-448 (1998); Selkoe, D.J. The cell biology of beta-amyloid precursor protein and presenilin in Alzheimer's disease. Trends in Cell Biol. 8, 447-453 (1998); Selkoe, D.J. Translating cell biology into therapeutic advances in Alzheimer's disease. Nature 399, A23-31 (1999); Wong, P.C., et al. An adverse property of a familial ALS-linked

10   SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. Neuron 14, 1105-1116 (1995); Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M. & Goedert, M. a-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. Proc. Natl. Acad. Sci. USA 95, 6469-6473 (1998); Hutton, M., et al. Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393, 702-705

15   (1998); Stone, M.J. Amyloidosis: a final common pathway for protein deposition in tissues. Blood 75, 531-545 (1990)). Whether branched polyamines might also prove efficacious in a variety of inherited disorders where the accumulation of abnormal proteins is a hallmark of the illness remains to be established; these genetic maladies include heritable forms of prion disease, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, frontotemporal dementia, Pick's disease and

20   amyloidosis, as well as the triplet repeat diseases including Huntington's disease, spinal cerebellar ataxias and myotonic dystrophy (Fu, Y.-H., et al. An unstable triplet repeat in a gene related to myotonic muscular dystrophy. Science 255, 1256-1259 (1992); Group, T.H.s.D.C.R. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72, 971-983 (1993)). Compounds identified via assays of the invention such as branched polyamines will find

25   utility in preventing or delaying the onset of these genetic diseases where carriers can often be identified decades in advance of detectable neurologic or systemic dysfunction.

The invention is based on the discovery that several dendritic polycations, including the starburst dendrimers Superfect™ (QIAGEN®, Valencia, CA), polyamidoamide (PAMAM), and the hyperbranched polycation polyethyleneimine (PEI), were surprisingly found to eliminate PrP^{Sc} from

30   cultured scrapie-infected neuroblastoma cells. These highly-branched, polycationic compounds provide a novel class of therapeutic agents to combat prion diseases and other degenerative disease including the amyloidoses. The removal of PrP^{Sc} is dependent on both the concentration of dendritic polymer and length of exposure. Dendritic polymers were able to clear PrP^{Sc} at concentrations which were not cytotoxic. Repeated exposures to heat-degraded starburst PAMAM dendrimer or PEI caused a dramatic reduction

35   in PrP^{Sc} levels which persisted for a month even after removal of the compound. Dendritic polycations

-32-

did not appear to destroy purified PrP^{Sc} in vitro, and therefore may act through a generalized mechanism. Dendritic polycations ▮▮▮ent a class of compounds which can be used ▮▮erapeutic agents in prion diseases and other disorders involving insoluble protein deposits, such as the amyloidoses.

5

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy

10 with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight-average molecular weight, temperature is in degrees Centigrade; and pressure is at or near atmospheric.

15

METHODS AND MATERIALS

Chemicals. High molecular weight PEI was purchased from Fluka. DOTAP cationic lipid was purchased from Boehringer Mannheim and SuperFect transfection reagent was purchased from QIAGEN®. All other compounds were purchased from Sigma-Aldrich. All test compounds were dissolved in water at stock concentration of 3 mg/ml and filtered through a Millipore 0.22 m m filter.

20

Cultured cells. Stock cultures of ScN2a cells were maintained in MEM with 10% FBS, 10% Glutamax (Gibco BRL), 100 U penicillin, and 100 mg/ml streptomycin (supplemented DME). Immediately prior to addition of test compounds, the dishes were washed twice with fresh supplemented DME media. After exposure to test compounds, dishes were drained of media and cells were harvested

25 by lysis in 0.25-1 ml 20 mM Tris pH 8.0 containing 100 mM NaCl, 0.5% NP-40, and 0.5% sodium deoxycholate to obtain a total protein concentration of 1 mg/ml measured by the BCA assay. Nuclei were removed from the lysate by centrifugation at 2000 rpm for 5 min. For samples not treated with proteinase K, 40 μl of whole lysate (representing 40 μg total protein) was mixed with an equal volume of 2x SDS reducing sample buffer. For proteinase K digestion, 20 μg/ml proteinase K (Boehringer

30 Mannheim) (total protein:enzyme ratio = 50:1) was added, and the sample was incubated for 1 h at 37°C. Proteolytic digestion was terminated by the addition of Pefabloc to a final concentration of 5 mM. One ml samples were centrifuged at 100,000 x g for 1 h at 4°C, the supernatants were discarded, and the pellets were resuspended in 80 μl of reducing SDS sample buffer for SDS-PAGE.

Brain homogenates. Brain homogenates from RML scrapie-affected CD-1 mice (10% (w/v) in sterile water) were pre███ by repeated extrusion through syringe needl█ ██ successively smaller size, from 18 to 22 gauge. Nuclei and debris were removed by centrifugation at 1000 x g for 5 min. The bicinchoninic acid (BCA) protein assay (Pierce) was used to determine protein concentration.

5 Homogenates were adjusted to 1 mg/ml protein in 1% NP-40. For reactions, 0.5 ml homogenate was incubated with 25 ml 1.0 M buffer (sodium acetate for pH 3-6 and Tris acetate for pH 7-10) plus or minus 10 ml of polyamine stock solution (3 mg/ml) for 2 h at 37°C with constant shaking. The final pH value of each sample was measured directly with a calibrated pH electrode (Radiometer Copenhagen). Following incubation, each sample was neutralized with an equal volume 0.2 M HEPES pH 7.5

10 containing 0.3 M NaCl and 4% Sarkosyl. Proteinase K was added to achieve a final concentration of 20 μg/ml, and samples were incubated for 1 h at 37°C. Proteolytic digestion was terminated by the addition of Pefabloc to a final concentration of 5 μM. Ten μl of digested brain homogenate was mixed with equal volume 2x SDS sample buffer and analyzed by SDS-PAGE followed by Western blotting.

15 Western blotting. Following electrophoresis, Western blotting was performed as previously described (Scott, M., et al. Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. Cell 59, 847-857 (1989)). Samples were boiled for 5 min and cleared by centrifugation for 1 min at 14,000 rpm in aBeckman ultrafuge. SDS-PAGE was carried out in 1.5 mm, 12% polyacrylamide gels(Laemmli, U.K. Cleavage of structural proteins during the assembly

20 of the head of bacteriophage T-4. Nature 227, 680-685 (1970)). Membranes were blocked with 5% non-fat milk protein in PBST (calcium- and magnesium-free PBS plus 0.1% Tween 20) for 1 h at room temperature. Blocked membranes were incubated with primary RO73 polyclonal antibody (to detect MoPrP) (Serban, D., Taraboulos, A., DeArmond, S.J. & Prusiner, S.B. Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins. Neurology 40, 110-117 (1990)) or 3F4 monoclonal antibody

25 (to detect MHM2 PrP) (Kascsak, R.J., et al. Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. J. Virol. 61, 3688-3693 (1987)) at 1:5000 dilution in PBST overnight at 4°C. Following incubation with primary antibody, membranes were washed 3 x 10 min in PBST, incubated with horseradish peroxidase-labeled secondary antibody (Amersham Life Sciences) diluted 1:5000 in PBST for 30 to 60 min at 4°C and washed again for 3 x 10 min in PBST. After chemiluminescent

30 development with ECL reagent (Amersham) for 1 min, blots were sealed in plastic covers and exposed to ECL Hypermax film (Amersham). Films were processed automatically in a Konica film processor.

EXAMPLE 1A

Branched polyamines inhibit formation of nascent PrP^{Sc} and induce clearance of pre-existing PrP^{Sc}

Western blots were probed with 3F4 monoclonal antibody which recognizes newly expressed MHM2 PrP. ScN2a cells were exposed to SuperFect for 3 h and harvested 3 d after removal of SuperFect. Gells were run on both undigested, control sample and a sample subjected to limited proteolysis. The samples were run in separate lanes 1-6 with a control and limited proteolysis sample for each of the 6 lanes as follows: Lane 1: DOTAP-mediated transfection. Lane 2: 30 μg/ml SuperFect, 5 μg pSPOX MHM2. Lane 3: 75 μg/ml SuperFect, 5 μg pSPOX MHM2. Lane 4: 150 μg/ml SuperFect, 5 μg pSOX MHM2. Lane 5: 150 μg/ml SuperFect, 10 μg pSPOX MHM2. Lane 6: No addition of either transfection reagent or DNA. Forty μl of undigested brain homogenate was used in these studies while those samples subjected to limited digestion with proteinase K were concentrated 25-fold prior to SDS-PAGE. One ml of the digest were centrifuged at 100,000 x g for 1 h at 4°C and the pellets suspended in 80 μl of SDS sample buffer prior to SDS-PAGE followed by Western blotting. Apparent molecular weights based on migration of protein standards are 34.2, 28.3, and 19.9 kDa.

All of the control lanes 1-6 show multiple bands as expected. However, of the samples subjected to limited proteolytic only lane 1 shows bands. Unexpectedly, all of the partially digested sample lanes 2-5 show no bands and as expected no bands in the partially digested lane 6. These results show the effect of using SuperFect in clearing PrP^{Sc}.

EXAMPLE 1B

The blot described above was stripped of antibody, exposed to labeled R073 and redeveloped. The antibody 3F4 used in Example 1 binds to PrP^C but not to PrP^{Sc}. However, R073 binds to PrP^{Sc} and PrP^C. Lanes 1, 2 and 3 show decreasing amounts of PrP^{Sc} and lanes 4 and 5 show no detectable PrP^{Sc}.

EXAMPLE 2A

Gels were run on undigested controls 1-4 and as above, samples subjected to limited proteolysis. The lanes were as follows: Lane 1: No SuperFect. Lane 2: 30 μg/ml SuperFect. Lane 3: 75 μg/ml SuperFect. Lane 4: 150 μg/ml SuperFect. ScN2a cells were exposed to SuperFect for 3 h and harvested 3 d after removal of SuperFect. Apparent molecular weights based on migration of protein standards are 33.9, 28.8, and 20.5 kDa. In that each sample was tested after the same time period the results show the dose-dependent effect of SuperFect on PrP^{Sc} removal. Lanes 1, 2 and 3 show decreasing amounts of PrP^{Sc} and lane 4 shows no detectable PrP^{Sc}.

EXAMPLE 2B

To determine th● e-dependent effect of SuperFect three differe● els with four lanes each were prepared and run as follows: ScN2a cells were exposed to 7.5 μg/ml: SuperFect (lanes 1-4), PEI (average molecular weight ~60,000)(lanes 5-8), or PAMAM, generation 4.0 (lanes 9-12). Time of exposure times for each polyamine: 0 hours (lanes 1, 5, and 9), 4 hours (lanes 2, 6, and 10), 8 hours (lanes 3, 7, and 11), 16 hours (lanes 4, 8, and 12). All samples were subjected to limited proteolysis to measure PrP^{Sc}. Apparent molecular weights based on migration of protein standards are 38, 26, and 15 kDa. Lanes of each of the three panels show decreasing amounts of PrP^{Sc}.

EXAMPLE 3

In this example four panels A,B, C and D were created with panels having three double (control and test) lanes each. ScN2a cells were exposed to 1.5 μg/ml: (A) SuperFect, (B) PEI (average molecular weight ~60,000), (C) PAMAM, generation 4.0, or (D) no addition. Cells were harvested: Lane 1, before addition; Lane 2, immediately following 1 week continuous exposure to test compounds; and Lane 3, three weeks after removal of test compounds. Minus (-) symbol denotes undigested, control sample and plus (+) symbol designates sample subjected to limited proteolysis. Apparent molecular weights based on migration of protein standards are 33.9, 28.8, and 20.5 kDa. Test lanes 3 in panel A showed slight PrP^{Sc} after three weeks and test lanes 3 in panels B and C showed no detectable PrP^{Sc} whereas PrP^{Sc} was present in all lanes in panel D.

EXAMPLE 4A

Four separate gels were run to demonstrate the effect of adding chloroquine would have on PrP^{Sc} levels. The lanes 1 control and 3 where chloroquine was added show clear bands for PrP^{Sc} whereas lanes 2 and 4 with no chloroquine show barely detectable amounts of PrP^{Sc}. The four lanes were prepared as follows: ScN2a cells were treated Lane 1: Control media. Lane 2: 7.5 μg/ml PEI (average molecular weight ~60,000). Lane 3: PEI plus 100 μM chloroquine. Lane 4: PEI plus 30 μM NH$_4$Cl. Chloroquine and NH$_4$Cl were added 1 h prior to addition of PEI. Cells were harvested 16 hours after addition of PEI. All samples shown were subjected to limited proteolysis to measure PrP^{Sc}. Apparent molecular weights based on migration of protein standards are 38, 26, and 15 kDa.

EXAMPLE 4B

Eight lanes with SuperFect (+SF) and eight lanes without SuperFect (-SF) were prepared. Lanes 1-8 of each group had an adjusted pH of 3.6,, 4, 5, 6, 7, 8, 9 and 9.6. In vitro mixture of crude mouse brain homogenates with SuperFect under a range of pH conditions was performed as described in methods (measured final pH of each sample denoted above the lanes). Addition of 60 μg/ml SuperFect

-36-

denoted as "+SF" and control with no addition as "-SF". All samples shown were subjected to limited proteolysis to measure PrP^{Sc}. Apparent molecular weights based on migration of protein standards are 30 and 27 kDa. All lanes of the -SF group showed PrP^{Sc} present. Lanes 3-8 of the +SF group showed PrP^{Sc}. However, lanes 1 and 2 with respective pH levels of 3.6 and 4.0 showed very slight detectable

5   PrP^{Sc}. The results show that the ability of a blanched polycation such as SuperFect to clear PrP^{Sc} is pH dependent.

EXAMPLE 5

Sixteen different lanes were prepared as described. Lanes 1 and 2 were control lanes and each of

10   lanes 3-16 contained a different compound as tested in Table 1. The test compounds were all polyamines. Thus, the results show removal of PrP^{Sc} from brain homogenate in vitro by various polyamines. Samples were incubated with polyamines at pH 3.6 and processed as described in Methods. Each polyamine was tested at 60 μg/ml concentration. Lanes 1 and 2: control. Lane 3: poly-(L)lysine. Lane 4: PAMAM, generation 0.0. Lane 5: PAMAM, generation 1.0. Lane 6: PAMAM, generation 2.0.

15   Lane 7: PAMAM, generation 3.0. Lane 8: PAMAM, generation 4.0. Lane 9: PAMAM-OH, generation 4.0. Lane 10: PPI, generation 2.0. Lane 11: PPI, generation 4.0. Lane 12: linear PEI. Lane 13: high MW PEI. Lane 14: low MW PEI. Lane 15: average MW PEI. Lane 16: SuperFect. All samples shown were subjected to limited proteolysis to measure PrP^{Sc}. Apparent molecular weights based on migration of protein standards are 30 and 27 kDa. Table 1. Removal of PrP^{Sc} by polymer compounds. IC$_{50}$ =

20   approximate concentration of polymer required to reduce PrP^{Sc} to 50% of control levels in ScN2a cells after exposure for 16 hours. All compounds were tested at 5 different concentrations. PrP^{Sc} levels were measured by densitometry of Western blot signals.

TABLE 1

(Note that Table 1 includes information
on the characteristics of compounds used but that
the list does not correspond directly to lanes 1-16)

Compound	Molecular Weight	Primary NH_2 groups	IC_{50} (ng/ml)
PAMAM generation 0.0	517	4	>10,000
PAMAM generation 1.0	1,430	8	>10,000
PAMAM generation 2.0	3,256	16	2,000
PAMAM generation 3.0	6,909	32	400
PAMAM generation 4.0	14,215	64	80
PAMAM-OH generation 4.0	14,279	0	>10,000
PPI generation 2.0	773	8	2,000
PPI generation 4.0	3,514	32	80
Low MW PEI	~25,000		2,000
Average MW PEI	~60,000		400
High MW PEI	~800,000		80
Linear PEI	~60,000		2,000
poly-(L)lysine	~60,000	>500	10,000
SuperFect			400

Lanes 7, 8, 11 and 13 showed the best results, i.e. best ability to clear PrP^{Sc} under these conditions. Specifically, PAMAM generation 4.0 in lane 8 showed the best ability to clear PrP^{Sc} under these conditions whereas PAMAM-OH generation 4.0 showed almost no detectable ability to clear PrP^{Sc} and was comparable to the control.

EXAMPLE 6

Transfection of PrP^{Sc} Expressing Cells with Dendrimer Compounds

Cells of neuronal origin expressing PrP^{Sc} were examined for the ability of compounds to suppress PrP^{Sc} formation.

Transfection Studies

Stock cultures of N2a and ScN2a cells were maintained in MEM with 10% FBS, 10% Glutamax (Gibco BRL), 100 U penicillin, and 100 μg/ml streptomycin. Cells from a single confluent 100 mm dish were trypsinized and split into 10 separate 60 mm dishes containing DME plus 10% FBS, 10% Glutamax, 100 U penicillin, and 100 μg/ml streptomycin (supplemented DME) one day prior to transfection. Immediately prior to transfection, the dishes were washed twice with 4 ml supplemented DME media and then drained.

For DOTAP-mediated transfection, 15 μg pSPOX MHM2 was resuspended in 150 μl sterile Hepes Buffered Saline (HBS) on the day of transfection. The DNA solution was then mixed with an equal volume of 333 μg/ml DOTAP (Boehringer Mannheim) in HBS in Falcon 2059 tubes and incubated at room temperature for 10 minutes to allow formation of DNA/lipid complexes. Supplemented DME

5   (2.5 ml) was added to the mixture, and this was then pipetted onto drained cell monolayers. The following day, the medium containing DNA/lipid was removed and replaced with fresh supplemented DME. Cells were harvested three days later.

For Superfect™-mediated transfections/exposures, Superfect™ with or without DNA was added to 1 ml supplemented DME in a Falcon 2059 tube to achieve the specific concentrations needed for each

10   experiment. This mixture was pipetted up and down twice and then onto drained cell monolayers. After exposure for the indicated times, the medium containing Superfect™ was removed and replaced with fresh supplemented DME. Cells were harvested at specified times after removal of Superfect™.

Exposures to PPI (DAB-Am-8, Polypropylenimine octaamine Dendrimer, Generation 2.0 Aldrich 46,072-9), Intact PAMAM (Starburst (PAMAM)Dendrimer, Generation 4.

15   Aldrich 41,244-9), PEI (Sigma), poly-(L)lysine (Sigma), and poly-(D) lysine (Sigma) were performed as described above for Superfect™.

Isolation of Protein from Treated Cells

Cells were harvested by lysis in 1.2 ml of 20 mM Tris pH 8.0 containing 100 mM NaCl, 0.5%

20   NP-40, and 0.5% sodium deoxycholate. Nuclei were removed from the lysate by centrifugation at 2000 rpm for 5 min. This lysate typically had a protein concentration of 0.5 mg/ml measured by the BCA assay. For samples not treated with proteinase K, 40 μl of whole lysate (representing 20 μg total protein) was mixed with 40 μl of 2x SDS sample buffer. For proteinase K digestion, 1 ml of lysate was incubated with 20 μg/ml proteinase K (total protein:enzyme ratio = 25:1) for 1 hr at 37°C. Proteolytic

25   digestion was terminated by the addition of 8 μl of 0.5M PMSF in absolute ethanol. Samples were then centrifuged for 75 min in a Beckman TLA-45 rotor at 100,000 x g at 4°C. The pellet was resuspended by repeated pipetting in 80 μl of 1X SDS sample buffer. The entire sample (representing 0.5 mg total protein before digestion) was loaded for SDS-PAGE.

30   Western Blot Analysis

Immunoreactive PrP bands from the DOTAP-mediated transfection were detected before and after digestion with proteinase K with monoclonal antibody 3F4. The construct used to express PrP^{Sc} in the ScN2a cells is MHM2 a chimeric construct that differs from wild-type (wt) MoPrP at positions 108 and 111 (Scott et al., (1992) Protein Sci. 1:986-997). Substitution at these positions with the

35   corresponding residues (109 and 112 respectively) from the Syrian hamster (SHa) PrP sequence creates

an epitope for 3F4 (Kascsak et al., (1987) J. Virol. 61:3688-3693), which does not recognize endogenous wt MoPrP in ScN2a cells and hence facilitates specific detection of the transgene by Western blot.

Following electrophoresis, Western blotting was performed as previously described (Scott et al., (1989) Cell 59:847-857). Samples were boiled for 5 minutes and cleared by centrifugation for 1 minute at 14,000 rpm in a Beckman ultrafuge. SDS-PAGE was carried out in 1.5 mm, 12% polyacrylamide gels (Laemmli (1970) Nature 227:661-665). Membranes were blocked with 5% nonfat milk protein in PBST (calcium- and magnesium-free PBS plus 0.1% Tween 20) for 1 hour at room temperature. Blocked membranes were incubated with primary RO73 polyclonal or 3F4 monoclonal antibody at a 1:5000 dilution in PBST overnight at 4 °C.

Following incubation with primary antibody, membranes were washed 3 x 10 minutes in PBST, incubated with horseradish peroxidase-labeled secondary antibody (Amersham Life Sciences) diluted 1:5000 in PBST for 25 minutes at room temperature and washed again for 3x 10 minutes in PBST. After chemiluminescent development with ECL reagent (Amersham) for 1 minute, blots were sealed in plastic covers and exposed to ECL Hypermax film (Amersham). Films were processed automatically in a Konica film processor.

In contrast to DOTAP-transfected cells, ScN2a cells transfected with varying concentrations of Superfect™ and DNA did not appear to contain protease-resistant MHM2. Close scrutiny revealed that, prior to protease digestion, Superfect™-transfected samples express MHM2 bands which are not seen in the background pattern of the control sample. These observations indicate that MHM2 PrP was successfully expressed using Superfect™ transfection reagent, but conversion of MHM2 PrP^C to protease-resistant MHM2 PrP^{Sc} was inhibited by Superfect™.

To examine whether Superfect™ had affected levels of preexisting PrP^{Sc} in ScN2a cells, the Western blot probed with 3F4 antibody was reprobed with polyclonal antibody RO73, which is able to recognize endogenous MoPrP. Remarkably, Superfect™ caused the disappearance of preexisting MoPrP^{Sc} from ScN2a cells in a dose-dependent manner. After treatment with Superfect™, PrP^{Sc} could not be detected in the nuclear fraction, pellet, supernatant, or media. The concentration of Superfect™ required to fully remove preexisting PrP^{Sc} with a three hour exposure was 300 μg/ml, whereas 30 μg/ml was sufficient to interfere with the formation of new MHM2 PrP^{Sc} within the same time frame.

Length of exposure dramatically influenced the ability of Superfect™ to remove PrP^{Sc} from ScN2a cells. Whereas a 3 hour exposure to 150 μg/ml Superfect™ significantly lowered PrP^{Sc} levels in ScN2a cells, exposure for 10 min to the same dose of Superfect™ did not affect PrP^{Sc} levels. When ScN2a cells were exposed to 2 μg/ml Superfect™ continuously for 1 week, PrP^{Sc} disappeared completely.

The conditions tested did not appear to be toxic for the cells. Neither 150 µg/ml Superfect™ for 3 hrs nor 2 µg/ml Superfect™ continuously for 1 week caused any obvious changes in cell morphology, viability, or growth as judged by phase contrast microscopy.

5   EXAMPLE 7

Elimination of PrP^{Sc} by repeated exposures to Superfect™

The duration in the reduction in PrP^{Sc} levels after exposure to Superfect™ was examined, and it was shown that this reduction could persist for extended periods after removal of Superfect™. Following the exposure of ScN2a cells to a single dose of 150 µg/ml Superfect™ for 3 hrs, PrP^{Sc} levels remained

10   low for one week, but returned to near baseline levels after 3 weeks in culture without Superfect™

In contrast, when ScN2a cells were exposed to 4 separate doses of Superfect™ over the course of 16 days, very little PrP^{Sc} could be detected 4 weeks after the final exposure to Superfect™. This result offers hope that prolonged exposure to Superfect™ may lead to long term cure of scrapie infection in cultured cells.

15

                                           EXAMPLE 8

Superfect™ does not destroy PrP^{Sc} directly

The dendrimer Superfect™ was used to determine if it could exert a similar inhibitory effect on PrP^{Sc} in either crude brain homogenates or purified PrP 27-30 rods.

20   Brain homogenates from normal and scrapie-affected Syrian hamsters (10% (w/v) in sterile PBS) were prepared by repeated extrusion through syringe needles of successively smaller size, from 18 to 22 gauge. Nuclei and debris were removed by centrifugation at 1000 x g for 10 min. The bicinchnoninic acid (BCA) protein assay (Pierce) was used to determine protein concentration. Homogenates were adjusted to 10 mg/ml protein with PBS and 50 µl was added to 450 µl of lysis buffer containing 100 mM NaCl, 1

25   mM EDTA, 0.55% sodium deoxycholate, 0.55% Triton X-100, and 50 mM Tris-HCl pH 7.5. This mixture was then incubated with 0-300 µg/ml Superfect™ for 3 hrs at 37 °C and then centrifuged for 10 min at 14,000 rpm in a Beckman Ultrafuge. The pellet was resuspended in 450 µl lysis buffer without Superfect™. Proteinase K (Boehringer Mannheim) was added to achieve a final concentration of 20 µg/ml, and thus the ratio of total protein/enzyme was 50:1. Samples were incubated for 1 h at 37 °C.

30   Proteolytic digestion was terminated by the addition of 8 µl of 0.5 M PMSF in ethanol. Samples were then centrifuged for 75 min in a Beckman TLA-45 rotor at 100,000 x g at 4 °C. Undigested samples (10 µl) were mixed with an equal volume of 2x SDS sample buffer. For digested samples, the pellet was resuspended by repeated pipetting in 100 µl 1x SDS sample buffer. Twenty µl (equivalent to 100 µg of total protein prior to proteinase K digestion) of each sample was loaded for SDS-PAGE.

-41-

PrP 27-30 rods were purified from scrapie-affected Syrian hamster brains and previously described (Prusiner et ●●● 1983) Cell 35:349-358). Purified rods (3.5 µg ●●) were incubated with or without 900 µg/ml Superfect™ in 100 µl supplemented DME. After 16 hrs at 37 °C, the suspension was centrifuged at 100,000 x g at 4 °C. The pellet was resuspended in 500 µl of buffer containing 1

5   mg/ml BSA, 100 mM NaCl, 1 mM EDTA, 0.55% sodium deoxycholate, 0.55% Triton X-100, and 50 mM Tris-HCl pH 7.5. Proteinase K was added to achieve a final concentration of 20 µg/ml. Samples were incubated for 1 h at 37 °C. Proteolytic digestion was terminated by the addition of 8 µl of 0.5 M Pefabloc (Boehringer Mannheim). Samples were then centrifuged for 75 min at 100,000 x g at 4 °C. Undigested samples (50 µl) were mixed with an equal volume of 2x SDS sample buffer. For digested

10   samples, the pellet was resuspended by repeated pipetting in 100 µl 1x SDS sample buffer. Forty µl of each sample was loaded for SDS-PAGE.

When Superfect™ was mixed with either crude homogenates of scrapie-affected Syrian hamsters or with purified Syrian hamster PrP 27-30, there was no significant change in the level of proteinase K-resistant PrP^{Sc}. These results suggest that the removal of PrP^{Sc} from ScN2a cells by Superfect™

15   depends on the presence of intact cellular machinery.

EXAMPLE 9

Clearance of PrP^{Sc} levels by other dendritic polycations

The Superfect™ compound is a high molecular weight component of heat-degraded PAMAM

20   Starburst dendrimers, which is a cationic, highly-branched, monodisperse polymers (Tang et al., (1996) Bioconjugate Chem. 7:703-714). To identify other potentially useful anti-prion therapeutic agents, we screened three other dendritic polycations and two linear cationic polymers for their ability to clear PrP^{Sc} from ScN2a cells. Among the dendritic macromolecules tested, polyetheleneimine (PEI) was the most potent, removing the majority of PrP^{Sc} from ScN2a cells after 3 hrs when used at a concentration of 10

25   µg/ml. Intact PAMAM displayed a potency comparable to Superfect™, removing approximately half of the detectable PrP^{Sc} when used at a concentration of 50 µg/ml. In contrast, the dendrimer polypropyleneimine (PPI), poly-(L)lysine, and the linear polycation poly-(D)lysine failed to reduce PrP^{Sc} levels at concentrations between 10-50 µg/ml. These results demonstrate that a branched polymeric architecture is required to clear PrP^{Sc}. Furthermore, exposure of ScN2a cells to either PEI or intact

30   PAMAM for one week at a concentration of 1.5 µg/ml completely removes PrP^{Sc}, effectively curing the cells of scrapie infection.

EXAMPLE 10
BRANCHED POLYAMINES CURE PRION-INFECTED NEUROBLASTOMA CELLS

The above Examples show that branched polyamines purged scrapie-infected neuroblastoma
(ScN2a) cells of PrP^{Sc}, the protease-resistant isoform of the prion protein. The ability of these compounds to eliminate PrP^{Sc} from ScN2a cells depended upon certain molecular characteristics. In particular, active compounds were highly branched and possessed a high surface density of primary amino groups. The most potent compounds identified were generation 4.0 polyamidoamide (PAMAM) and polypropyleneimine (PPI) dendrimers. Dendrimers are branched polyamines manufactured by a repetitive divergent growth technique, allowing the synthesis of successive, well-defined "generations" of homodisperse structures. The following experimental results demonstrate that branched polyamines cure prion-infected cells. We also determine the site and mechanism of action for these compounds.

MATERIALS AND METHODS

Chemical compounds. High molecular weight PEI was purchased from Fluka. SuperFect transfection reagent was purchased from QIAGEN®. All other polyamines were purchased from Sigma-Aldrich. Fluorescein-labeled PPI was synthesized as follows: 30 mg fluorescein isothiocyanate (FITC) was mixed with 1 mg PPI generation 4.0 in 2 ml absolute ethanol overnight at 4°C. We intentionally set up a 3:1 excess of PPI-to-FITC equivalent groups to minimize the production of multiple FITC conjugates per PPI. Labeled PPI was separated from residual, unreacted FITC using a 12 mm X 37 cm Sephadex P-2 column equilibrated in 0.15 mM NaCl buffer. Fractions were collected and analyzed by thin layer chromatography for single spots of fluorescence and amine content. Fluorescence was detected using a long wave UV lamp, and primary amines were detected by ninhydrin assay. Appropriate fractions were combined and lyophilized. The dry powder was brought up in sterile water, titrated to pH 7.0, diluted in 5% glucose, 5 mM HEPES pH 7.4, and filtered through a 0.2 Fm polycarbonate membrane. FITC concentration of this stock solution was 44.1 FM, as measured by UV spectroscopy with an absorbance maxima at 489 nm. Final PPI concentration was 50 FM.

Cultured cells. Cultures of ScN2a cells were maintained in DME pH 7.4 with 10% FBS, 10% Glutamax (Gibco BRL), 100 U/ml penicillin, and 100 Fg/ml streptomycin (supplemented DME). Cultures were split 1:10 weekly, and fed fresh medium twice weekly. Cytotoxicity after treatment with polyamines was assessed in ScN2a cells by four methods: (1) examination of morphology under phase contrast microscopy, (2) observation of growth curves and cell counts for three weeks after treatment, (3) vital staining of living cells with 0.4% trypan blue (Sigma-Aldrich), and (4) assay of dehyrogenase enzymes with 93-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). For the dehyrogenase assay, cells in 96-well plates were incubated with 0.5 mg/ml MTT (Sigma-Aldrich) in supplemented DME for 4 hrs. Media was then aspirated and cells were dissolved in isopropanol

containing 50 mM HCl. Converted MTT was measured by absorbance at 570 nm. For ScN2a cells treated with either P⬤ M or PPI generation 4.0 continuously for 1 w⬤ LD_{50} ~50 Fg/ml.

To prepare samples for infectivity assays, 100 mm plates (Falcon) of confluent cells were washed with 3 x 5 ml PBS, scraped into 2 ml PBS, and homogenized by repeated extrusion through a 26 gauge needle. Prion infectivity was determined by intracerebral inoculation of 30 Fl cell homogenate into Tg(MoPrP)4053 mice. Mice were observed for clinical signs of scrapie, and a subset of diagnoses were confirmed by neuropathological examination.

To prepare samples for SDS-PAGE, plates were drained of media and adherent cells were lysed in 1 ml 20 mM Tris pH 8.0, 100 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate. Samples were adjusted to obtain a total protein concentration of 1 mg/ml measured by the bicinchoninic (BCA) assay (Pierce). Nuclei were removed from the lysate by centrifugation at 2000 rpm for 5 min. For samples not treated with proteinase K, 10 Fl of lysate was mixed with an equal volume of 2 x SDS reducing sample buffer. For proteinase K digestion, 20 Fg/ml proteinase K (Boehringer Mannheim) (total protein:enzyme ratio = 50:1) was incubated with 1 ml lysate for 1 hr at 37 °C. Proteolytic digestion was terminated by the addition Pefabloc (Boehringer Mannheim) to a final concentration of 5 mM. Samples were then centrifuged at 100,000 x g for 1 hr at 4 °C and the pellet fractions were resuspended in 80 Fl of reducing SDS sample buffer. Twenty microliter samples were loaded per lane on 1 mm 12% Tris glycine SDS-PAGE gels (Novex).

Mixture of brain homogenates and purified prions with polyamines in vitro. Brain homogenates from scrapie-infected rodents (10% (w/v) in sterile water) were prepared by repeated extrusion through syringe needles of successively smaller size, from 18 to 22 gauge. Nuclei and debris were removed by centrifugation at 1000 x g for 5 min. Homogenates were adjusted to 1 mg/ml protein in 1% NP-40. For incubations with PPI, 50 Fl 1 mg/ml brain homogenate was mixed with 450 Fl 1% NP40, 50 mM sodium acetate pH 3.0 (final measured pH = 3.6) plus or minus 60 Fg/ml PPI generation 4.0 and shaken constantly for various periods at 37 °C.

Purified prions were prepared as described previously, utilizing both proteinase K digestion and sucrose gradient sedimentation, and resuspended in 1% NP-40, 1 mg/ml BSA. For pH studies, 475 Fl of 0.5 Fg/ml purified RML PrP27-30 in 1% NP-40, 1 mg/ml BSA was mixed with 25 Fl 1M buffers from pH 3-8 (sodium acetate for pH 3-6 and Tris acetate for pH 7-8) plus or minus 60 Fg/ml PPI generation 4.0 for 2 hrs at 37 °C with constant shaking. The final pH value of each sample was measured directly with a calibrated pH electrode (Radiometer Copenhagen). For compound screening, 475 Fl of 0.5 Fg/ml purified RML PrP27-30 in 1% NP-40, 1 mg/ml BSA was mixed with 25 Fl 1M sodium acetate pH 3.0 plus 60 Fg/ml polyamine for 2 hrs at 37 °C with constant shaking.

Following incubations, each sample was neutralized with an equal volume 0.2 M HEPES pH 7.5 containing 0.3 M NaCl and 4% Sarkosyl. Samples not treated with proteinase K were mixed with equal volume 2x SDS sample buffer. For proteinase K digestion, samples were incubated with 20 Fg/ml proteinase K (Boehringer Mannheim) (total protein:enzyme ratio = 25:1) for 1 hr at 37°C. Proteolytic

digestion was terminated by the addition of 8 μl of 0.5M PMSF in absolute ethanol. Digested samples were then mixed with ⬤ volumes 2 x SDS sample buffer. All sampl ⬤ re boiled for 5 min prior to electrophoresis. SDS-PAGE was performed on 1.5 mm 12% polyacrylamide gels.

5   Western blotting. Following electrophoresis, Western blotting was performed as previously described. Membranes were blocked with 5% non-fat milk protein in PBST (calcium- and magnesium-free PBS plus 0.1% Tween 20) for 1 hr at room temperature. Blocked membranes were incubated with 1 Fg/ml recombinant, humanized Fab d13 in PBST for 1 hr at 4 °C. Following incubation with primary Fab d13, membranes were washed 3 x 10 min in PBST, incubated with horseradish peroxidase-labeled

10   anti-human Fab secondary antibody (ICN) diluted 1:5000 in PBST for 45 min at room temperature and washed again for 4 x 10 min in PBST. After chemiluminescent development with ECL reagent (Amersham) for 1-5 min, blots were sealed in plastic covers and exposed to ECL Hypermax film (Amersham). Films were processed automatically in a Konica film processor.

15   Negative stain electron microscopy. Sample preparation was done on carbon-coated 600 mesh copper grids that were glow-discharged for 30 sec prior to staining. Five microliter samples were adsorbed to grids for 30 - 60 sec, washed with 2 drops of 0.1 M and 0.01 M ammonium acetate each, and stained with 2 drops of freshly filtered 2% ammonium molybdate or uranyl acetate. After drying, samples were viewed in a Jeol JEM 100CX II electron microscope at 80 kV at a standard magnification

20   of 40,000. The magnification was calibrated using negatively-stained catalase crystals.

      Confocal microscopy. Confocal images were obtained using a BioRad laser scanning confocal microscope (MRC-1024, Hercules, CA), outfitted with a Nikon Diaphot 200 microscope and a Helium/Neon laser. A 60X Nikon planAPO lens was used, with an additional software zoom function.

25   Laser power was set at 10%, and scanned with a slow speed across the sample. Individual laser lines confirmed the lack of "bleed through" between detection channels. The images were averaged with a Kalman filter (n=4).

      ScN2a cells were incubated with 3 Fg/ml PPI in supplemented DME for 4 weeks, and then cultured for an additional 2 weeks in polyamine-free medium. This transient exposure to PPI was not

30   cytotoxic (see Methods) and completely purged the cells of protease-resistant PrP^{Sc} (Figure 2A, lanes 2 and 4). In contrast, protease-sensitive PrP^{C} bands migrating between 32-38 kDa appear unaltered by PPI treatment (lane 2). Elimination of PrP^{Sc} appeared to be relatively specific since the steady-state levels of proteins in PPI-treated cells was similar to those in control ScN2a cells (Figure 2B). To assess the effect of PPI treatment on prion infectivity, homogenates prepared from polyamine-treated and

35   control ScN2a cells were inoculated into Tg(MoPrP)4053 mice. The average scrapie incubation time was 67 + 2 days for mice inoculated with control ScN2a cells and >120 days for mice inoculated with ScN2a cells treated with PPI ($n/n_0 = 0/10$) (Figure 2C). These incubation times indicate that the titer of

infectious prions in ScN2a cells was reduced from ~10^7 LD_{50} units/100 mm plate to < 1 LD_{50} unit/plate by PPI treatment. T██████xposure to PPI completely eliminates prion i████vity from ScN2a cells.

Figure 2. Treatment of scrapie-infected neuroblastoma cells with polyamidoamide dendrimer. ScN2a cells were treated with 3 Fg/ml PPI generation 4.0 in supplemented DME or control media for 4 weeks . After 2 additional weeks of culture in compound-free media, cells were harvested for analysis. (Panel A) PrP immunostain with recombinant Fab d13 was performed as described in Methods. Apparent molecular weights based on migration of protein standards are 30 and 27 kDa. (Panel B) Silver stain was performed as previously described. Apparent molecular weights based on migration of protein standards are 49, 36, 25, and 19 kDa. (Panels A and B) Samples subjected to SDS-PAGE were assigned lanes as follows: (1 and 3) control cells, (2 and 4) PPI-treated cells. Lanes (1) and (2) contain undigested lysates, and lanes (3) and (4) contain lysates subjected to limited proteolysis with proteinase K. (Panel C) Infectivity bioassay of cell homogenates in Tg(MoPrP)4053 indicator mice: (Filled circles) control cells, (Open squares) PPI-treated cells.

EXAMPLE 11

BRANCHED POLYAMINES ACT DIRECTLY ON PURIFED RML PRIONS

Having established that branched polyamines reduce prion infectivity, we sought to identify the mechanism by which these compounds eliminate PrP^{Sc}. An initial objective was to determine the molecular target of branched polyamines. Above is described an in vitro assay which was used to show that these compounds could render PrP^{Sc} protease-susceptible when mixed directly with crude brain homogenates. A similar assay was carried out with purified RML PrP27-30 prions to determine whether or not the molecular target of branched polyamines was present in this highly purified preparation. PrP^{Sc} in purified preparations of RML PrP27-30 were rendered protease-sensitive by branched polyamines with a similar acidic pH optimum (Figure 3A) and structure-activity profile (Figure 3B) as previously obtained in crude brain homogenates. These results indicate that the molecular target of branched polyamines must either be: (1) PrP^{Sc} itself; (2) an acid-induced unfolding intermediate of PrP^{Sc}; or (3) a very tightly bound, cryptic molecule which copurifies with PrP^{Sc}.

Figure 3. Mixture of purified prions with branched polyamines in vitro. (Panel A) Samples containing 0.5 Fg/ml purified mouse RML PrP27-30 in 1 mg/ml BSA were incubated for 2 hrs at 37°C with 60 Fg/ml PPI generation 4.0 or control buffer at different pH values as indicated. (Panel B) Samples containing 0.5 Fg/ml purified mouse RML PrP27-30 in 1 mg/ml BSA were incubated for 2 hrs at 37°C with various polyamines: (1-2) control, (3) poly-(L)lysine, (4) PAMAM 0.0, (5) PAMAM 1.0, (6) PAMAM 2.0, (7) PAMAM 3.0, (8) PAMAM 4.0, (9) PAMAM-OH 4.0, (10) PPI 2.0, (11) PPI 4.0, (12) linear PEI, (13) high MW PEI, (14) low MW PEI, (15) average MW PEI, (16) Qiagen SuperFect. All compounds were tested at a concentration of 60 Fg/ml. (Panels A and B) Western immunoblotting was performed with recombinant Fab d13. Apparent molecular weights based on migration of protein standards are 30 and 27 kDa.

EXAMPLE 12
PrP? SCEPTIBILITY TO PPI-INDUCED CONF◯ ATIONAL
CHANGE IS SEQUENCE- AND STRAIN-SPECIFIC

Although the PrP sequence is well conserved among mammals, a small number of amino acid

5   substitutions appear to hinder prion transmission across species. Furthermore, prions can exist as different phenotypic strains that yield distinct incubation times, neuropathology, and distribution of PrP^{Sc} upon infection of susceptible hosts. In certain cases, these phenotypic differences can be correlated with differences in the conformation of PrP^{Sc}. An effort was made to determine whether different species and strains of rodent prions, which presumably contain different conformations of PrP^{Sc}, vary in their

10   susceptibility to branched polyamines. Homogenates were prepared from the brains of rodents infected with one of several Syrian hamster (SHa), mouse (Mo), or artificial prion strains. Individual samples were mixed with 60 Fg/ml PPI generation 4.0 in vitro for 2 hrs at 37°C, neutralized, and subjected to limited proteolysis. The results indicate that susceptibility to PPI dendrimer is dependent on both prion strain and PrP sequence (Figure 4A).

15   The varying susceptibility of different strains is most clearly illustrated by the 6 mouse strains analyzed (paired lanes 7-12). Mo(RML), Mo(22a), and Mo(139A) were susceptible to PPI-induced conformational change (paired lanes 7, 9, and 11, respectively). In contrast, Mo(Me7) and Mo(87V) were resistant (paired lanes 8 and 10, respectively); and Mo(C506) was marginally susceptible to PPI-induced conformational change (paired lane 12).

20   The effect of PrP sequence can be seen by comparing the relative susceptibilities of SHa(RML), MH2M(RML), and Mo(RML). Whereas Mo(RML) was susceptible to PPI-induced conformational change (paired lane 7), SHa(RML) was resistant (paired lane 4). MHM2(RML) displayed an intermediate level of susceptibility to PPI (paired lane 5); MHM2 is a chimeric PrP molecule in which amino acids 94 to 188 of the mouse sequence have been replaced by the corresponding SHa residues.

25   Thus, SHaPrP^{Sc} appears to be more resistant to PPI-induced conformational change than MoPrP^{Sc}.

We investigated whether the varying susceptibilities to PPI displayed by different strains and species of prions might be caused by kinetic differences. To test this possibility, we incubated samples of each prion isolate with PPI generation 4.0 for varying periods of time. Even after incubation with PPI for 3 days, PrP^{Sc} in samples of resistant isolates did not become more susceptible to protease digestion

30   (for example, Figure 4B). Thus, the differences in susceptibilities of different prion strains and sequences are not caused simply by differences in the kinetics of PrP^{Sc} unfolding.

Figure 4. Treatment of different prion strains with polypropyleneimine in vitro. (Panel A) Samples containing 1% (w/v) various brain homogenates were incubated for 2 hrs at 37°C with 60 Fg/ml PPI generation 4.0. Paired lanes are designated as follows: (1) SHa(Sc237), (2) SHa(139H), (3)

35   SHa(drowsy), (4) SHa(RML), (5) Tg(MH2M)Pmp$^{0/0}$(RML), (6) Tg(PrP106) Pmp$^{0/0}$(RML), (7) mouse(RML), (8) mouse(Me7), (9) mouse(22a), (10) mouse(87V), (11) mouse(139A), (12) mouse(C506). Minus (-) symbol denotes undigested, control sample and plus (+) symbol designates sample subjected to limited proteolysis by proteinase K. (Panel B) Samples containing 1% (w/v)

EXAMPLE 12

PrP^{Sc} CEPTIBILITY TO PPI-INDUCED CONFO ATIONAL CHANGE IS SEQUENCE- AND STRAIN-SPECIFIC

Although the PrP sequence is well conserved among mammals, a small number of amino acid
5   substitutions appear to hinder prion transmission across species. Furthermore, prions can exist as different phenotypic strains that yield distinct incubation times, neuropathology, and distribution of PrP^{Sc} upon infection of susceptible hosts. In certain cases, these phenotypic differences can be correlated with differences in the conformation of PrP^{Sc}. An effort was made to determine whether different species and strains of rodent prions, which presumably contain different conformations of PrP^{Sc}, vary in their
10   susceptibility to branched polyamines. Homogenates were prepared from the brains of rodents infected with one of several Syrian hamster (SHa), mouse (Mo), or artificial prion strains. Individual samples were mixed with 60 Fg/ml PPI generation 4.0 in vitro for 2 hrs at 37°C, neutralized, and subjected to limited proteolysis. The results indicate that susceptibility to PPI dendrimer is dependent on both prion strain and PrP sequence (Figure 4A).

15   The varying susceptibility of different strains is most clearly illustrated by the 6 mouse strains analyzed (paired lanes 7-12). Mo(RML), Mo(22a), and Mo(139A) were susceptible to PPI-induced conformational change (paired lanes 7, 9, and 11, respectively). In contrast, Mo(Me7) and Mo(87V) were resistant (paired lanes 8 and 10, respectively); and Mo(C506) was marginally susceptible to PPI-induced conformational change (paired lane 12).

20   The effect of PrP sequence can be seen by comparing the relative susceptibilities of SHa(RML), MH2M(RML), and Mo(RML). Whereas Mo(RML) was susceptible to PPI-induced conformational change (paired lane 7), SHa(RML) was resistant (paired lane 4). MHM2(RML) displayed an intermediate level of susceptibility to PPI (paired lane 5); MHM2 is a chimeric PrP molecule in which amino acids 94 to 188 of the mouse sequence have been replaced by the corresponding SHa residues.
25   Thus, SHaPrP^{Sc} appears to be more resistant to PPI-induced conformational change than MoPrP^{Sc}.

We investigated whether the varying susceptibilities to PPI displayed by different strains and species of prions might be caused by kinetic differences. To test this possibility, we incubated samples of each prion isolate with PPI generation 4.0 for varying periods of time. Even after incubation with PPI for 3 days, PrP^{Sc} in samples of resistant isolates did not become more susceptible to protease digestion
30   (for example, Figure 4B). Thus, the differences in susceptibilities of different prion strains and sequences are not caused simply by differences in the kinetics of PrP^{Sc} unfolding.

Figure 4. Treatment of different prion strains with polypropyleneimine in vitro. (Panel A) Samples containing 1% (w/v) various brain homogenates were incubated for 2 hrs at 37°C with 60 Fg/ml PPI generation 4.0. Paired lanes are designated as follows: (1) SHa(Sc237), (2) SHa(139H), (3)
35   SHa(drowsy), (4) SHa(RML), (5) Tg(MH2M)Prnp$^{0/0}$(RML), (6) Tg(PrP106) Prnp$^{0/0}$(RML), (7) mouse(RML), (8) mouse(Me7), (9) mouse(22a), (10) mouse(87V), (11) mouse(139A), (12) mouse(C506). Minus (-) symbol denotes undigested, control sample and plus (+) symbol designates sample subjected to limited proteolysis by proteinase K. (Panel B) Samples containing 1% (w/v)

mouse(RML) or SHa(Sc237) were incubated at 37°C with 60 Fg/ml PPI generation 4.0 or control buffer for the time periods indicated. (Panels A and B) Western immunoblotting was performed with recombinant Fab d13. Apparent molecular weights based on migration of protein standards are 30 and 27 kDa. Prion strains were obtained from the following sources: SHa(Sc237), Mo(139A), and Mo(Me7)

5   from R. Kimberlin; SHa(139H) from R. Carp; SHa(drowsy) from R. Marsh; Mo(RML) from W. Hadlow; Mo(22a) and Mo(87V) from A. Dickenson; Mo(C506) from J. Gibbs. SHa(RML) was obtained by passaging Mo(RML) directly into Syrian hamsters. The pasage histories of other strains have been reviewed [Ridley, 1996 #4337].

EXAMPLE 13

10   ## BRANCHED POLYAMINES ASSIST PrP^{Sc} DISAGGREGATION

The existence of prion strains resistant to branched polyamines suggests that PrP^{Sc} molecules in these strains might exist in conformations which are more resistant to denaturation than PrP^{Sc} molecules in polyamine-susceptible strains. To test this hypothesis, an examination was made of the effect of adding urea to SHa(Sc237) brain homogenate treated with and without PPI generation 4.0. In the

15   presence of urea, PrP^{Sc} was more susceptible to protease digestion in samples treated with PPI, whereas no difference in protease-resistance could be detected in the absence of urea (Figure 5A). Thus, additional denaturation enables PrP^{Sc} molecules in a resistant strain to become susceptible to branched polyamines. This result suggests that the general mechanism of action of branched polyamines might be to assist PrP^{Sc} denaturation. Consistent with this concept, branched polyamines render PrP^{Sc} protease-

20   sensitive more efficiently at lower pH values (Figure 4A) and higher temperatures (Figure 5B). Furthermore, polyamine-treated PrP^{Sc} did not regain protease-resistance after prolonged neutralization (Figure 5C) or dialysis (data not shown). Finally, we excluded the possibility that acidification might be required only to activate the dendrimer by demonstrating that pre-acidified PPI generation 4.0 could not render PrP^{Sc} protease-sensitive at neutral pH (Figure 5D).

25   To visualize the effect of branched polyamines on prions, we examined the ultrastructure of purified prion rods treated in vitro with PPI generation 4.0. By electron microscopy, Mo(RML) PrP27-30 rods were disaggregated after incubation for 2 hrs at 37°C with PPI (Figure 6B). In contrast, SHa(237) PrP27-30 rods remained intact after treatment with PPI (Figure 6D).

To investigate further the mechanism of polyamine-induced disaggregation of PrP^{Sc}, we

30   performed a kinetic study in vitro using purified RML PrP27-30 and various concentrations of PPI. The results indicate that polyamine-induced PrP^{Sc} disaggregation is not a catalytic process, and requires a stoichiometry of approximately 1 PPI molecule per 5 RML PrP27-30 molecules in purified prion preparations.

Figure 5. Denaturation of PrP^{Sc} is enhanced by polypropyleneimine. (Panel A) Samples

35   containing 1% (w/v) SHa(Sc237) brain homogenates were incubated for 2 hrs at 37°C with 60 Fg/ml PPI generation 4.0 or control buffer, plus various concentrations of urea as indicated. All samples were subjected to limited proteolysis. (Panel B) Samples containing 0.5 Fg/ml purified mouse RML PrP27-30 in 1 mg/ml BSA were incubated for 2 hrs at various temperatures with 60 Fg/ml PPI generation 4.0.

Paired lanes are designated as follows: (1) 4°C (2) 20°C, (3) 37°C. Minus (-) symbol denotes undigested, control s⬤ and plus (+) symbol designates sample subj⬤ to limited proteolysis. (Panel C) Samples containing 1% mouse(RML) brain homogenate in 1% NP40, 50 mM sodium acetate pH 3.6 were incubated at 37°C for 2 hrs with either: (odd lanes) no addition or (even lanes) 60 Fg/ml

5   PPI. All samples were neutralized with an equal volume 0.2 M HEPES pH 7.5 containing 0.3 M NaCl and 4% Sarkosyl. (1 and 2) samples not subjected to protease digestion, (3 and 4) samples immediately subjected to limited proteinase K digestion, (5 and 6) samples incubated at pH 7.5 for an additional 16 hrs at 37°C before proteinase K digestion. (Panel D) Samples containing 1% mouse(RML) brain homogenate were treated in the following manner: (1) control sample at pH 3.6, (2) mixed with 60 Fg/ml

10   PPI at pH 3.6 for 2 hrs, (3) mixed with 60 Fg/ml PPI at pH 7.0 for 2 hrs, (4) incubated alone at pH 3.6 for 2 hrs and then mixed with 60 Fg/ml PPI (pre-titrated to pH 7.0) for 10 min, (5) incubated alone at pH 7.0 for 2 hrs and then mixed with 60 Fg/ml PPI (pre-titrated to pH 3.0) for 10 min. All incubations were carried out at 37°C. Minus (-) symbol denotes undigested, control sample and plus (+) symbol designates sample subjected to limited proteolysis by proteinase K. (Panels A-D) Western

15   immunoblotting was performed with recombinant Fab d13. Apparent molecular weights based on migration of protein standards are 30 and 27 kDa.

Figure 6. Ultrastructure of purified prion rods treated with polyproplyeneimine in vitro. Samples of purified 100 Fg/ml PrP27-30 in 0.1% NP40, 50 mM sodium acetate pH 3.0 buffer were

20   shaken countinously for 2 hrs at 37°C. Samples were then prepared for negative stain electron microscopy as described in Methods. (A) mouse(RML), (B) mouse(RML) plus 60 Fg/ml PPI generation 4.0, (C) SHa(Sc237), (D) SHa(Sc237) plus 60 Fg/ml PPI generation 4.0. Negative stain used was 2% uranyl acetate; scale bar = 100 nm.

25   EXAMPLE 14
                          PPI ACCUMULATES IN LYSOSOMES
        Branched polyamines apparently require acidic conditions to render PrP^{Sc} protease-sensitive when mixed with brain homogenates or purified prions in vitro (Figure 3A). However, these compounds successfully cure living culture media buffered at pH 7.4 (Figure 2). One possible explanation for this

30   discrepancy is that branched polyamines might co-localize with prions within an acidic intracellular compartment. PrP^{Sc} has previously been shown to accumulate in lysosomes. Therefore, we sought to determine whether branched polyamines localize to this same compartment. We incubated N2a cells with fluorescein-labeled PPI and LysoTracker Red, and performed dual channel confocal microscopy to compare the localization of the two compounds. Our results indicate that fluorescein-labeled PPI

35   accumulates in the lysosomes of living cells (Figure 7A, 7B and 7C).
        Figure 7. Polypropyleneimine localizes to lysosomes N2a cells grown to 50% confluence on coverslips were incubated for 4hrs with 3 FM FITC-PPI and 1 hr with 75 nM LysoTracker Red (Molecular Probes) in supplemented DME. Following incubation, coverslips were washed 3 times with

PBS, fixed with 2% paraformaldehyde in PBS, and mounted onto microscope slides with VECTASHIELD. Fluorescence confocal microscopy in green and red channels was performed as described in Methods. Scale bar = 5 Fm.

BRANCHED POLYAMINES AS THERAPEUTIC AGENTS

A major finding of the above Examples is that branched polyamines eliminate prion infectivity in chronically infected living cells. This is believed to be the first class of compounds shown to cure an established prion infection. Polyene antibiotics, anionic dyes, sulphated dextrans, anthracylines, porphyrins, phthalocyanines, dapsone, and a synthetic $-breaker peptide all prolong scrapie incubation times in vivo, but only if administered very early in the course of infection.

The unique ability of branched polyamines to cure an established prion infection in cells indicates that they can be used for a therapy in animals, even when administered after the onset of symptoms. However, two factors could potentially limit the use of these compounds as therapeutic agents against prion diseases. Branched polyamines might not act on all strains of prions, and they might not cross the blood-brain barrier. The first possibility is suggested by our data which show that some strains and species of prions are more resistant than others to branched polyamine-induced disaggregation in vitro. It remains to be determined whether prion strains resistant to branched polyamine-induced disaggregation in vitro would also be resistant to treatment by these compounds in vivo. Treatment of more resistant strains might require therapy with branched polyamines in combination with another class of prion-directed compounds.

The second potential limitation of branched polyamines is that these highly charged comounds might not cross the blood-brain barrier. If this proves to be the case, branched polyamines could be delivered directly to the CSF through an intraventricular reservoir, or perhaps synthesized as prodrugs capable of crossing the blood-brain barrier. Preliminary studies indicate that continuous intraventricular infusion of PPI generation 4.0 is tolerated by FVB mice up to a total dose of approximately 2 mg/animal (data not shown).

MOLECULAR TARGET, MECHANISM, AND SITE OF ACTION

It is important to characterize the molecular and cellular mechanisms by which branched polyamines eliminate prions for two reasons. First, branched polyamines could potentially be used as research tools to study the cellular and structural biology of prions. Second, identifying the molecular target of branched polyamines would facilitate the design of other compounds more specifically directed against this target.

The ability of branched polyamines to render PrP^{Sc} protease-sensitive in purified preparations (Figures 3A and 3B) suggests that the molecular target of these compounds must either be (1) PrP^{Sc} itself; (2) an acid-induced unfolding intermediate of PrP^{Sc}; or (3) a very tightly bound, cryptic molecule which copurifies with PrP^{Sc}. If the molecular target is PrP, at least one of the polyamine binding sites must be contained within the amino acid sequence of the PrP106 deletion mutant, since PPI renders

PrP^{Sc}106 protease-sensitive (Figure 4A, lane 6). The 106 amino acids present in PrP106 are residues 89-140 and 177-231. PP██so renders a spontaneously protease-resistant,██mino acid-long PrP deletion mutant, PrP()23-88,)█-221), susceptible to protease-digestion, further confining the boundaries of at least one putative binding site to residues 89-140 and 222-231.

5   Several lines of evidence suggest that branched polyamines render PrP^{Sc} molecules protease-sensitive by dissociating PrP^{Sc} aggregates. (1) RML PrP27-30 prion rods treated in vitro with PPI become disaggregated, as judged by electron microscopy (Figures 6A and 6B). (2) Prion strains resistant to branched polyamines in vitro appear to be more amyloidogenic than polyamine-susceptible strains, as judged by neuropathology. (3) The ability of branched polyamines to render PrP^{Sc} protease-sensitive in

10   vitro is enhanced by conditions which favor PrP^{Sc} disaggregation. These conditions include lower pH (Figure 3A), higher temperature (Figure 5B), and the presence of urea (Figure 5A).

        Theoretically, it is possible that the mechanism by which branched polyamines remove PrP^{Sc} and prion infectivity from ScN2a cells does not relate to the ability of these compounds to disaggregate prions in vitro. However, this is unlikely because the relative potency of 14 different polyamines in eliminating

15   PrP^{Sc} from ScN2a cells exactly matches the relative ability of these same compounds to render PrP^{Sc} protease-sensitive in crude brain homogenates and purified preparations of RML PrP27-30 in vitro (Figure 3B). The structure-activity profile obtained from these studies indicates that polyamines become more potent at eliminating PrP^{Sc} as they become more branched and possess more surface primary amines. With PPI dendrimers, this effect reaches a plateau at the fourth generation; PPI generation 5.0 is

20   no more potent than PPI generation 4.0 at either removing PrP^{Sc} from cells or rendering PrP^{Sc} protease-sensitive in vitro. Homodisperse, uniform PPI and PAMAM dendrimers were more potent than the heterogeneous preparations of polyethyleneimine (PEI) or SuperFect, a heat-fractured dendrimer.

        We determined that the process by which PPI renders PrP^{Sc} protease-sensitive in vitro was not catalytic. Instead, this process appeared to require a fixed stoichiometric ratio of PPI to PrP^{Sc} of

25   approximately 1:5. The question was presented regarding how PPI could disaggregate prion rods stoichiometrically. One possible explanation is that individual amino groups on the surface of PPI might bind to PrP^{Sc} monomers or oligomers that exist in equilibrium with a large aggregate under acidic conditions. The dendrimer might then pry bound PrP^{Sc} molecules apart from the aggregate and/or prevent such molecules from reaggregating.

30   Several lines of evidence indicate that the cellular site of action of branched polyamines is secondary lysosomes. (1) Fluorescein-tagged PPI and PrP^{Sc} both localize to lysosomes (Figure 7). (2) The pH optimum of PrP^{Sc} disaggregation in vitro is <5.0. When cultured cells were studied with fluorescent acidotropic pH measurement dyes, secondary lysosomes were the most acidic cellular compartment detected, with pH values~4.4-4.5. (3) The lysosomotropic agent chloroquine attenuates the

35   ability of branched polyamines to eliminate PrP^{Sc} from ScNa cells. Our studies raise the possibility that lysosomal proteases normally degrade PrP^{Sc} in prion-infected cells at a slow rate, and that polyamines accelerate this process by disaggregating PrP^{Sc}.

OTHER APPLICATIONS OF BRANCHED POLYAMINES

Beyond their ⬤ ntial use as therapeutic agents and research to ⬤ branched polyamines might also be useful as prion strain typing reagents and/or prion decontaminants. Presently, typing of prion strains is time-consuming and requires the inoculation of samples into several strains of inbred animals to obtain incubation time and neuropathology profiles. In the Examples provided above it was shown that different species and strains of prions displayed varying susceptibilities to branched polyamine-induced disaggregation in vitro (Figure 4A). These results indicate that a polyamine-based in vitro protease digestion assay could be used as a simple and rapid diagnostic method for prion strain typing. Currently, it is very difficult to remove prions from skin, clothes, surgical instruments, foodstuffs, and surfaces. Standard prion decontamination requires either prolonged autoclaving or exposure to harsh protein denaturants such as 1N NaOH or 6M guanidine thiocyanate. Branched dendrimers are non-toxic and relatively inexpensive. These compounds are suitable for use as a sterilizing agent to limit the commercial and iatrogenic spread of prion disease.

EXAMPLE 15

Results provided here show that acidic conditions (<pH 5) enhance the ability of other compounds (e.g. dendrimers, SDS, and urea) to denature PrP^{Sc} and destroy prion infectivity. Specific results demonstrate that acidic conditions can be used to formulate effective prion disinfectants emphasizing the importance of acidic conditions..

THE EFFECTIVENESS OF 1% SDS as pH 3.4
to ELIMINTE PrP^{Sc} at 5 MINUTES OR 2 HOURS

A 2% homogenate of scrapie Sc237 brain in water was prepared by repeated extrusion through a 22 G needle. Nuclei were removed by centrifugation for 5 min at 1000 rpm. The clarified homogenate was diluted 2-fold and incubated for (Top panel of Figure 8A) 2 hrs or (Bottom panel of Figure 8B) 5 min at 37°C under the following conditions:

1. 1% NP40, 50 mM sodium acetate pH 7.0
2. 1% NP40, 0.5% acetic acid pH 3.4
3. 1% SDS, 50 mM sodium acetate pH 7.0
4. 1% SDS, 0.5% acetic acid pH 3.4
5. 1% Sarkosyl, 50 mM sodium acetate pH 7.0
6. 1% Sarkosyl, 0.5% acetic acid pH 3.4

Plus (+) lanes indicate samples subjected to limited proteolysis with 20 μg/ml proteinase K for 1 hr at 37°C, Minus (-) lanes indicate samples not subjected to proteolysis. All samples were boiled in SDS sample buffer for 5 min prior to SDS polyacrylamide gel eletrophoresis. Following transfer

toMillipore Immobilon transfer membrane, development of the immunoblot was performed with d13
primary Fab.

THE EFFECT OF TEMPERATURE
ON THE ABILITY OF 1% SDS
AT pH 3.4 TO ELIMINATE PrP^{Sc}

A 2% homogenate of scrapie Sc237 brain in water was prepared by repeated extrusion through a
22 G needle. Nuclei were removed by centrifugation for 5 min at 1000 rpm. The clarified homogenate
was diluted 2-fold and incubated for 5 min under the following conditions:

1.   1% SDS, 0.5% acetic acid pH 3.4 at 4°C
2.   1% SDS, 0.5% acetic acid pH 3.4 at 20°C
3.   1% SDS, 0.5% acetic acid pH 3.4 at 37°C
4.   1% NP40, 0.5% acetic acid pH 3.4 at 20°C

Plus (+) lanes of Figure 9 indicate samples subjected to limited proteolysis with 20 μg/ml
proteinase K for 1 hr at 37°C. Minus (-) lanes indicate samples not subjected to proteolysis. All
samples were boiled in SDS samples buffer for 5 min prior to SDS polyacrylamide gel electrophoresis.
Following transfer to Millipore Immobilon transfer membrane, development of the immunoblot was
performed with d13 primary Fab.

THE ABILITY OF ACIDIC CONDITIONS
TO ENHANCE UREA-MEDIATED
PrP^{Sc} DENATURATION

A 2% homogenate of scrapie Sc237 brain in water was prepared by repeated extrusion through a
22 G needle. Nuclei were removed by centrifugation for 5 min at 1000 rpm. The clarified homogenate
was diluted 2-fold and incubated for 2 hours with 0.5% NP40 plus urea and 50 mM sodium acetate
buffer (urea concentration and pH are indicated above Figure 10).

All samples were subjected to limited proteolysis with 20 μg/ml proteinase K for 1 hr at 37°C,
and boiled in SDS samples buffer for 5 min prior to SDS polyacrylamide gel electrophoresis. Following
transfer to Millipore Immobolin transfer membrane, developments of the immunoblot was performed with
d13 primary Fab.

EXAMPLE 16
SDS/ACETIC ACID FORMULATION

Samples of 1% Syrian hamster brain homogenate containing 10^7 LD$_{50}$ units prion infectivity/ml
were incubated with either 50 mM Tris acetate pH 7.0 or 0.5% acetic acid in the presence of either 1%
NP-40 or 1% SDS for 2 h at 37°C. Following incubation, each sample was inoculated intracerebrally

-53-

into 8 separate Syrian hamsters for a scrapie incubation time assay. The results are shown in the table below:

sample	LD_{50}/ml
1% NP40, 50 mM Tris acetate pH 7.0	10^7
1% NP40, 5% acetic acid, pH 3.6	10^7
1% SDS, 50 mM Tris acetate pH 7.0	10^5
1% SDS, 5% acetic acid, pH 3.6	$<10^2$

The above results clearly demonstrate that a formulation comprised of approximately 1% SDS and approximately 0.5% acetic acid is effective in inactivating prions. Such a formulation could provide for an extremely valuable commercial formulation due to the ready availability of both acetic acid and SDS. However, those skilled in the art will recognize that other effective acids and effective detergents with structure similar to SDS could be formulated to obtain the same or similar results. With respect to the acid component what is important is to create a formulation which keeps the pH of the formulation acidic and preferably below 5.0 and more preferably below 4.0. The detergent composition need not be SDS. For example, the sodium component of the detergent could be any cation such as calcium, lithium, potassium, magnesium etc. Further, the sulfate component could be substituted with chemically equivalent moieties. Sodium dodecyl sulfate includes a hydrocarbon component with eleven CH_2 groups terminated by a CH_3 group. Various other alkyl groups such as other straight chained, branched or cyclic groups could be utilized. The alkyl moiety could contain from 2 to 40 carbons and more preferably contains approximately 12 carbon atoms ± 6 carbon atoms. The formulation can be added to appropriate solvents, in appropriate concentrations. Further, the concentration of the formulation can be changed immediately prior to use and thus sold at a highly concentrated formulation or sold in a concentration ready for use without dilution with a solvent such as water or alcohol. Still further, as indicated above the formulations of the invention can be supplemented with appropriate antibacterial and/or antiviral components as well as components which inactivate other pathogens including parasites so that the final formulation is effective in killing or inactivating a wide range of infectious components.

EXAMPLE 17
EFFECT OF DETERGENT AND
pH ON PROTEASE-RESISTANT PrP^{Sc}

Samples of 1% Sc237-infected SHa brain homogenate were incubated for 15 min at 37°C with detergent at a range pH values as indicated. Fifty millimolar sodium acetate buffers were used to maintain pH values 3-6, and 50 mM Tris acetate buffers were used to maintain pH values 7-10. The final pH value of each sample denoted above the corresponding lanes was measured directly with a calibrated pH electrode (Radiometer Copenhagen). All samples were neutralized by addition of equal volume 4% Sarkosyl, 100 mM HEPES pH 7.5, 200 mM NaCl and subjected to limited proteolysis with 20 μg/ml proteinase K for 1 h at 37°C. Apparent molecular weights based on migration of protein

standards are 30 and 27 kDa. All samples were neutralized by addition of equal volume 4% Sarkosyl, 100 mM HEPES pH ●●●● 200 mM NaCl. Minus (-) symbol denotes un●●●●●ted, control sample and plus (+) symbol designates sample subjected to limited proteolysis with 20 μg/ml proteinase K for 1 h at 37°C. Apparent molecular weights based on migration of protein standards are 30 and 27 kDa. Figure

5   11 shows that SDS denatures PrPSc at pH <5 or pH ≥ 10.

EXAMPLE 18

CHARACTERIZATION OF PrP^{Sc}

DENATURATION MEDIATED BY ACIDIC SDS

10   Samples of 1% Sc237-infected SHa brain homogenate were incubated for 15 min at 37°C in 1% SDS plus (1) 50 mM Tris acetate pH 7.0, (2) 50 mM sodium acetate pH 3.6, (3) 50 mM glycine pH 3.7, and (4) 0.2% peracetic acid, pH 3.4. Following incubation, an equal volume of 4% Sarkosyl, 100 mM HEPES pH 7.5, 200 mM NaCl was added to neutralize each sample. Minus (-) symbol denotes undigested, control sample and plus (+) symbol designates sample subjected to limited proteolysis with

15   20 μg/ml proteinase K for 1 h at 37°C. Apparent molecular weights based on migration of protein standards are 30 and 27 kDa. Figure 12 shows that SDS denatures PrPSc under acidic conditions in different acidic buffers, including peracetic acid (a commonly used hospital disinfectant).

While the present invention has been described with reference to the specific embodiments

20   thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

That which is claimed ●   ●

1.   An antiseptic composition, comprising:

5   a solvent;

an acid component characterized by and present in a molarity so as to maintain the pH of the composition at about 5.0 or less; and

an active component characterized by reducing infectivity of an infectious protein when the composition is brought into contact with the infectious protein for about two hours or less.

10

2.   The composition of claim 1, wherein the solvent is selected from the group consisting of water, alcohol and a mixture thereof; and

wherein the acid component is characterized by maintaining the pH of the composition at about 4.0 or less.

15

3.   The composition of claim 1, wherein the acid component is of a type selected from the group consisting of a sulfuric acid, a hydrochloric acid, a nitric acid and a carboxylic acid present in a molarity so as to maintain a pH of about 4.0 or less and wherein the active component is characterized by reducing infectivity of infectious prions when the composition is brought into contact with the prions for

20   one hour or less at a temperature of 40°C or less at a pressure of about one atmosphere.

4.   The composition of claim 3, wherein the active component is selected from the group consisting of SDS, a branched polycation, a protein denaturant and urea.

25   5.   The composition of claim 1, wherein the infectious protein is a naturally occurring protein conformationally altered from its normal non-infectious conformation and wherein the conformationally altered protein is a protein selected from the group consisting of APP, $A\beta$ peptide, a Lewy body protein, α-1-antichymotrypsin, non-$A\beta$ component, presenillin 1, presenillin 2, apoE and PrP^{Sc}.

30

6.   The composition of claim 1, wherein the solvent is selected from the group consisting of water, alcohol and a mixture thereof present in an amount of 1% to 99.99% by weight and wherein the active component is present in an amount of about 0.001% to 10% weight.

35

7.   The composition of claim 6, further comprising:
a detergent in an amount of about 1% to 20% by weight;
an antibacterial compound in an amount of about 0.1% to 20% by weight;
an antiviral compound in an amount of 0.1% to 20% by weight; and
an antifungal compound in an amount of 0.1% to 20% by weight.

8.   The composition of claim 1, wherein the active component is selected from the group consisting of a protein denaturant, an inorganic salt, an organic solvent, a detergent and a branched polycation.

9.   The composition of claim 8, wherein the active component is a protein denaturant selected from the group consisting of urea, guanidine, guanidine hydrochloride, beta-mercaptoethanol, dithiothreitol (DTT), and a chaotrope.

10.   The composition of claim 8, wherein the active component is an inorganic salt selected from the group consisting of lithium bromide, thiocyanate, patassium thiocyanate, sodium iodide, ammonium chloride, EDTA (metal chelator), lithium ion and salts thereof, and formic acid and salts thereof.

11.   The composition of claim 8, wherein the active component is an organic solvent selected from the group consisting of formamide, dimethylformamide, dichloro- and trichloroacetic acids and their salts, and trifluroethanolamine (TFE).

12.   The composition of claim 8, wherein the active component is a detergent selected from the group consisting of sodium dodecyl sulfate (SDS) (also known as lauryl sulfate, sodium salt -- other salts are also useful including lithium and potassium salts, sodium cholate, sodium deoxycholate, octylglucoside, dodecyldimethylamine oxide, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dodecyltriethylammonium bromide (DTAB), cetyltrimethylammonium bromide (CTAB), polyoxyethylene-p-isooctylphenyl ether (e.g. Triton X-20, Triton X-100, Triton X-114).

13.   The composition of claim 8, wherein the active component is a branched polycation selected from the group consisting of polyamidoamide (PAMAM) dendrimers, polypropyleneimine (PPI) dendrimers, polyethyleneimine (PEI) dendrimers, poly (4'-aza-4'-methylheptamethylene D-glycaramide), polyamidoamines, fragments and variants of any of a-e.

-57-

14.   A method of treating a material to inactivate infections prions, comprising the steps of: contacting the material with the composition as claimed in any claims 1-13; and allowing the composition to remain in contact with the material for a time and under conditions which inactivate infectious prions.

15.   The method of claim 14, wherein the material is selected from the group consisting of a pharmaceutical gel capsule, a gel coated tablet, a blood extender or blood replacement solution, a surgical implant, a bandage, a suture, a dental implement, a dental sponge, a surgical sponge, a candy containing a gelatin such as a caramel candy, a marshmallow, or a mint such as an Altoid ® mint, doughnut glaze, fruit juice, wine, beer, sour cream, yogurt, cottage cheese, ice cream, margarine, and chewing gum.

16.   A method of forming a gelatin capsule, comprising:
extracting hooves from an ungulate;
treating the hooves so as to create a gelatin material;
treating the gelatin with an acid of a type and in an amount as to reduce the pH of the gelatin to 4.0 or less;
adding to the gelatin an active component characterized by destroying infectivity of infectious prions in about two hours or less.

17.   A gelatin capsule produced by the method of claim 16.

18.   The gelatin capsule as claimed in claim 17, having therein a compound selected from the group consisting of a pharmaceutically active drug, a vitamin and a nutriceutical.

19.   A method of sterilizing an object, comprising the steps of:
contacting the object with an antiseptic composition comprised of
a solvent;
an acid component characterized and present in a molarity so as to maintain the pH of the composition at about 5.0 or less; and
an active component characterized by reducing infectivity of an infectious protein when the composition is brought into contact with the infectious protein for about two hours or less.

20.   The method of claim 19, wherein the object is selected from the group consisting of a surgical instrument, a diagnostic instrument, a clonoscope, a sigmoioscope, a bronchoscope, a gastroscope, a dental instrument, a catheter, an operating room, a cell culture, a chromotographic column, and gelatin material obtained from a cow.

21. The method of claim 19, wherein the active component is selected from the group consisting of polyprop███e imine, polyethyleneimine (PEI) poly(4'-aza███ethylheptamethylene D-glucaramide), polyamidoamines and variants or fragments thereof, SDS, and urea.

5   22. A method of producing a pharmaceutically active protein in sterile form, comprising:
culturing cells designed to express a protein of interest;
extracting the protein of interest; and
contacting the protein of interest with an antiseptic composition comprising
a solvent;

10   an acid component characterized and present in a molarity so as to maintain the pH of the composition at about 5.0 or less; and

an active component characterized by reducing infectivity of an infectious protein when the composition is brought into contact with the infectious protein for about two hours or less.

15   23. The method of claim 22, wherein the infectious protein is a prion.

Figure 1

1   2   3   4   1   2   3   4
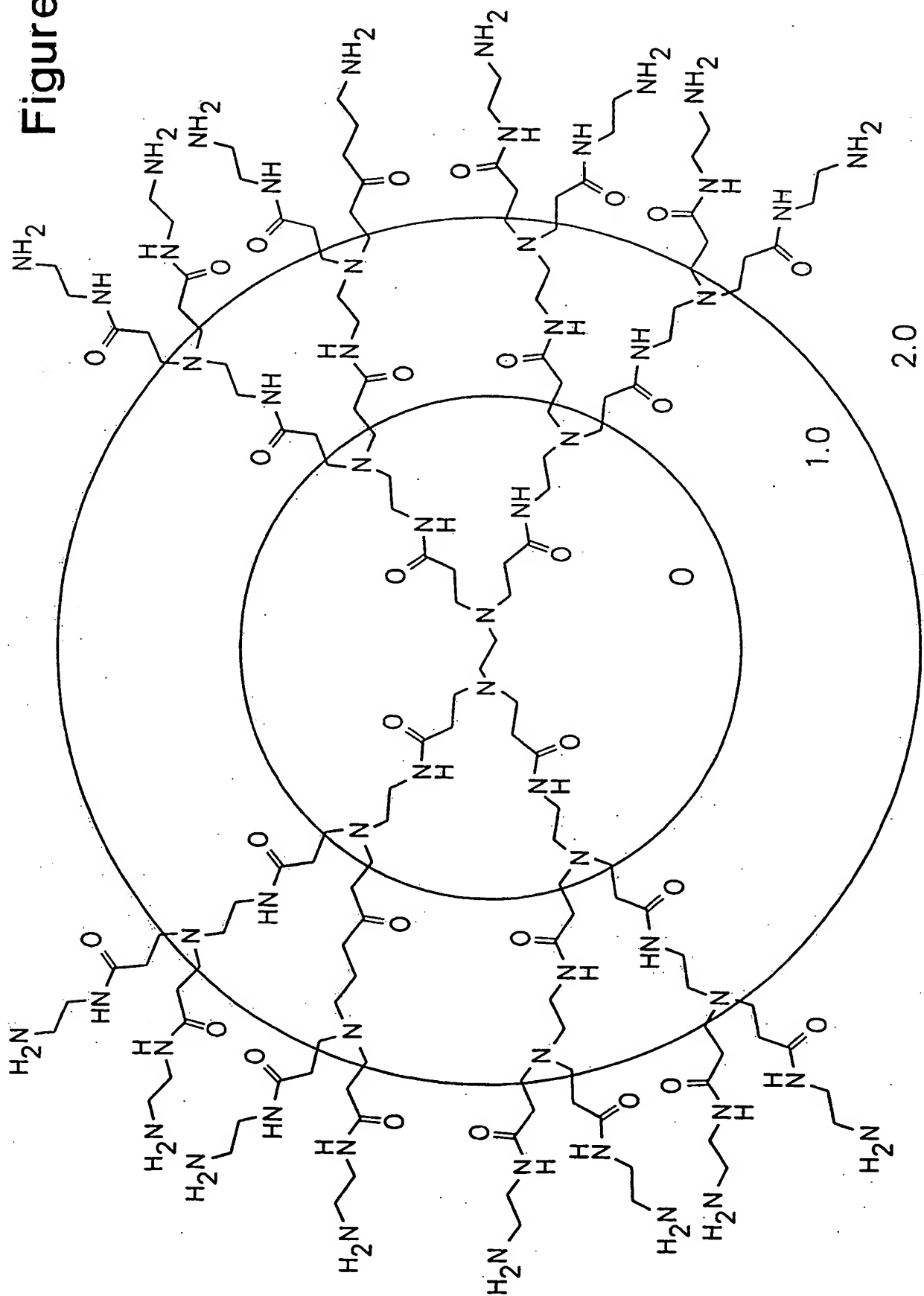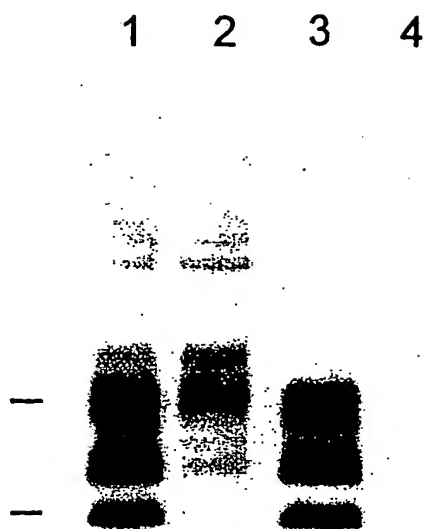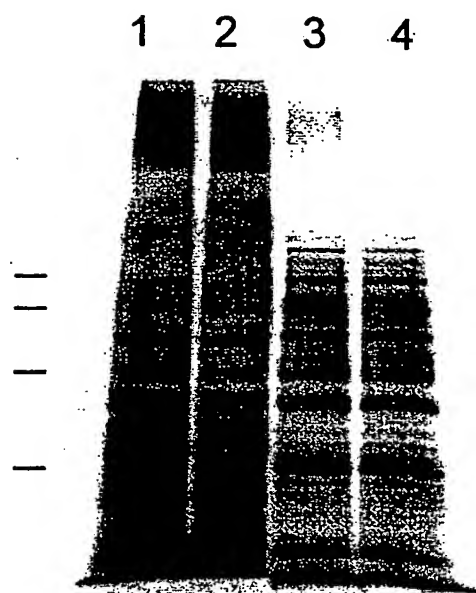
Figure 2A

Figure 2B
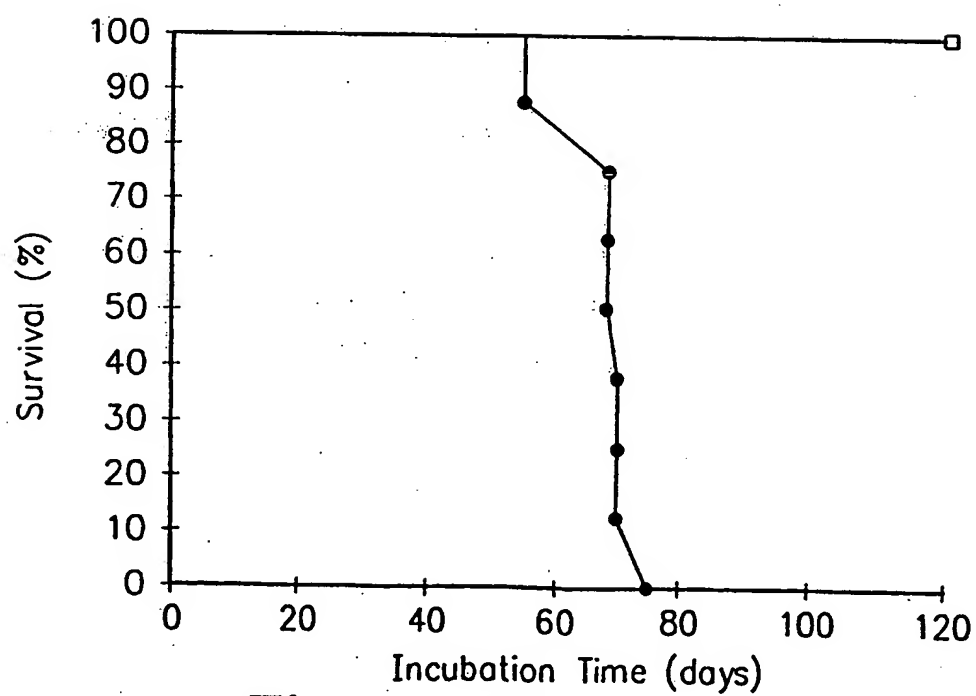
Figure 2C

Figure 3A
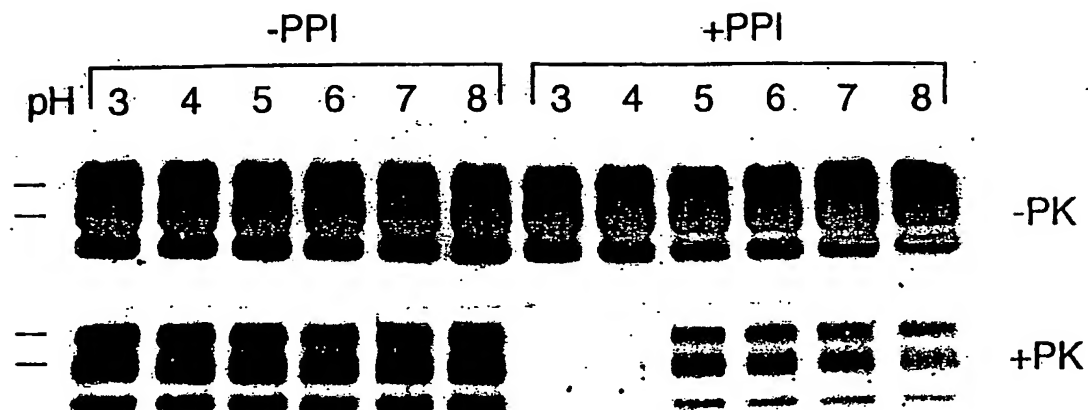
Figure 3B

Figure 4A

Figure 4B

Figure 5A

[urea, M]: 0 1 2 3 4 5

-PPI

+PPI

Figure 5B

1   2   3

\- + \- + \- +

Figure 5C

1 2 3 4 5 6

Figure 5D

1   2   3   4   5

\- + \- + \- + \- + \- +
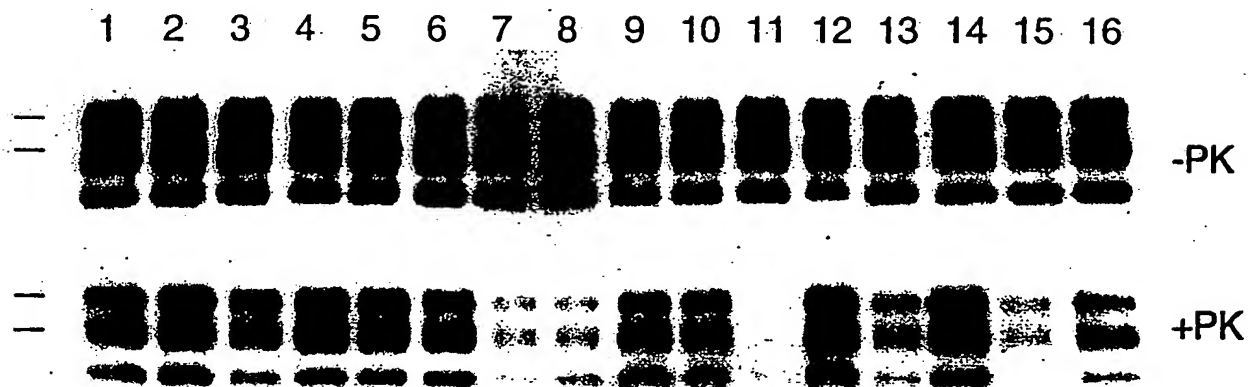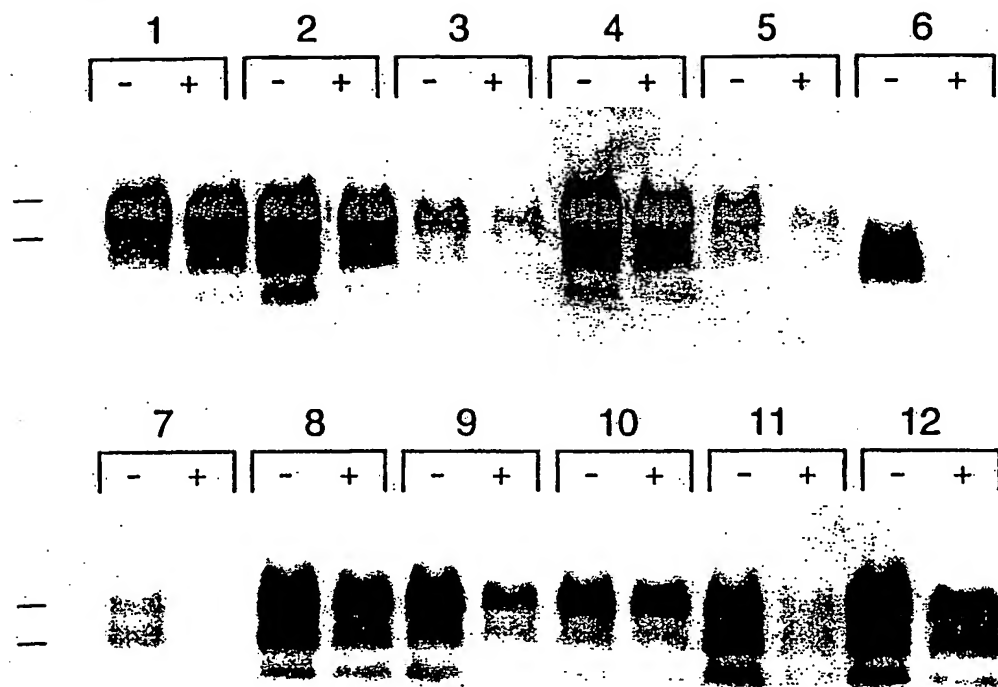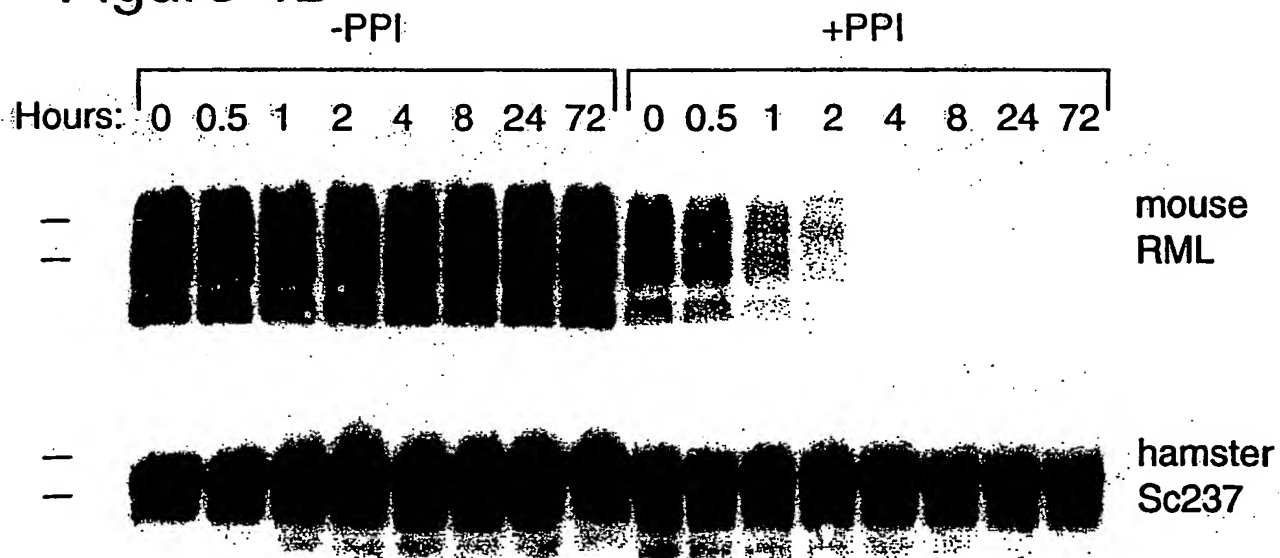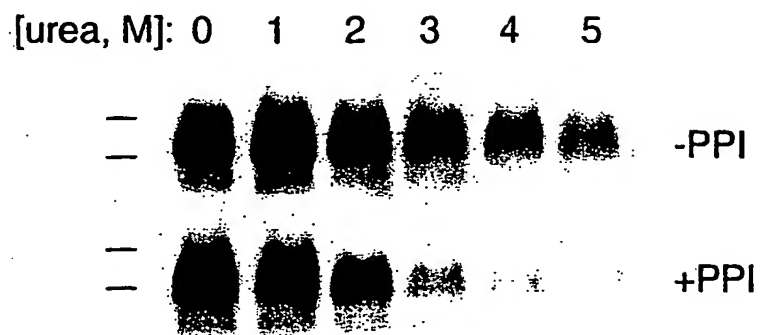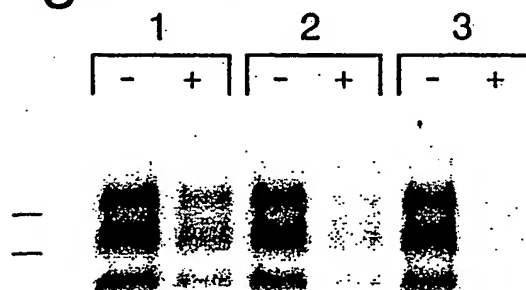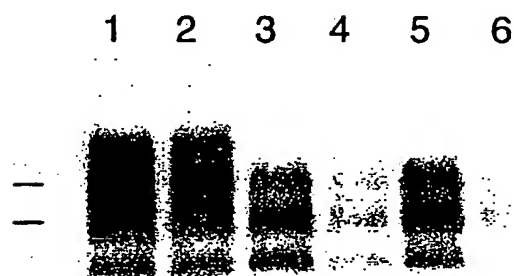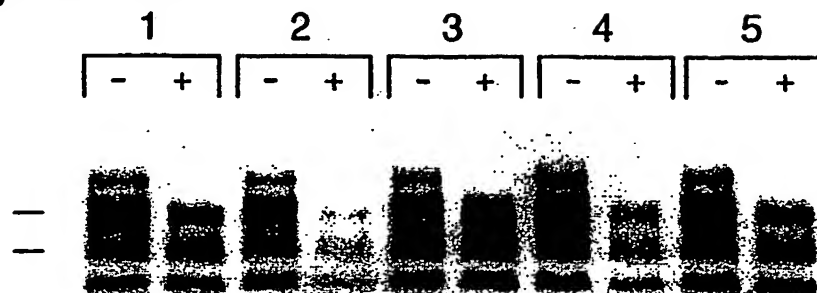
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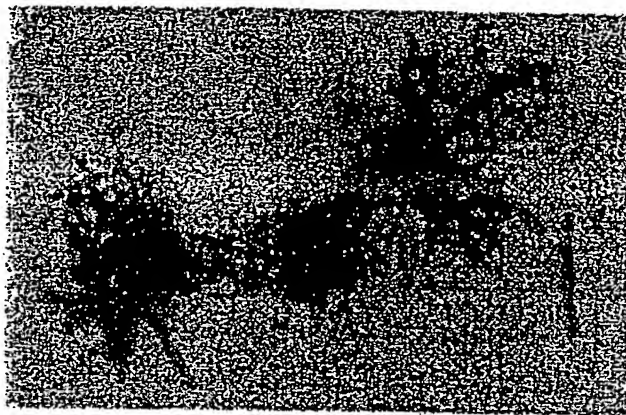
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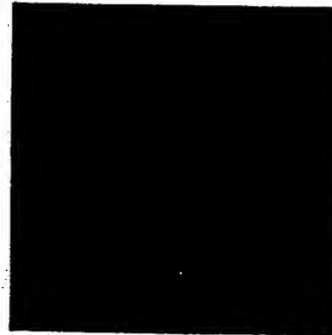
Sc237+PPI
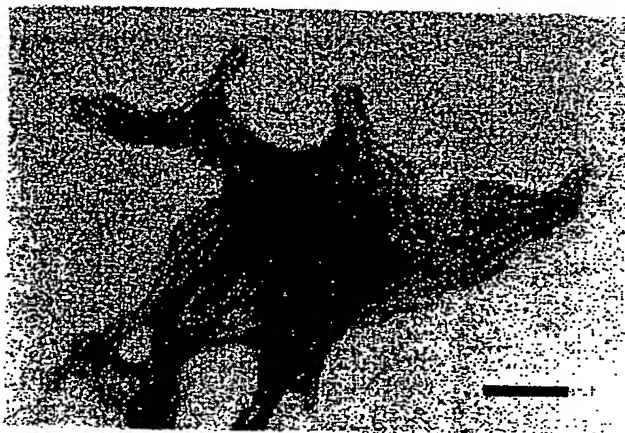
Figure 6B
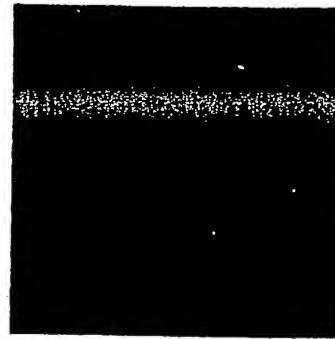
FITC-PPI

Figure 7A

LysoTracker

Figure 7B

Both

Figure 7C

Figure 8A

Figure 8B

Figure 9

Figure 10

Figure 11

pH   3.5 4.0 4.5 5.0 6.0 7.0 8.0 9.0 10.0

1% NP40

1% SDS

2% SDS

4% SDS

Figure 12

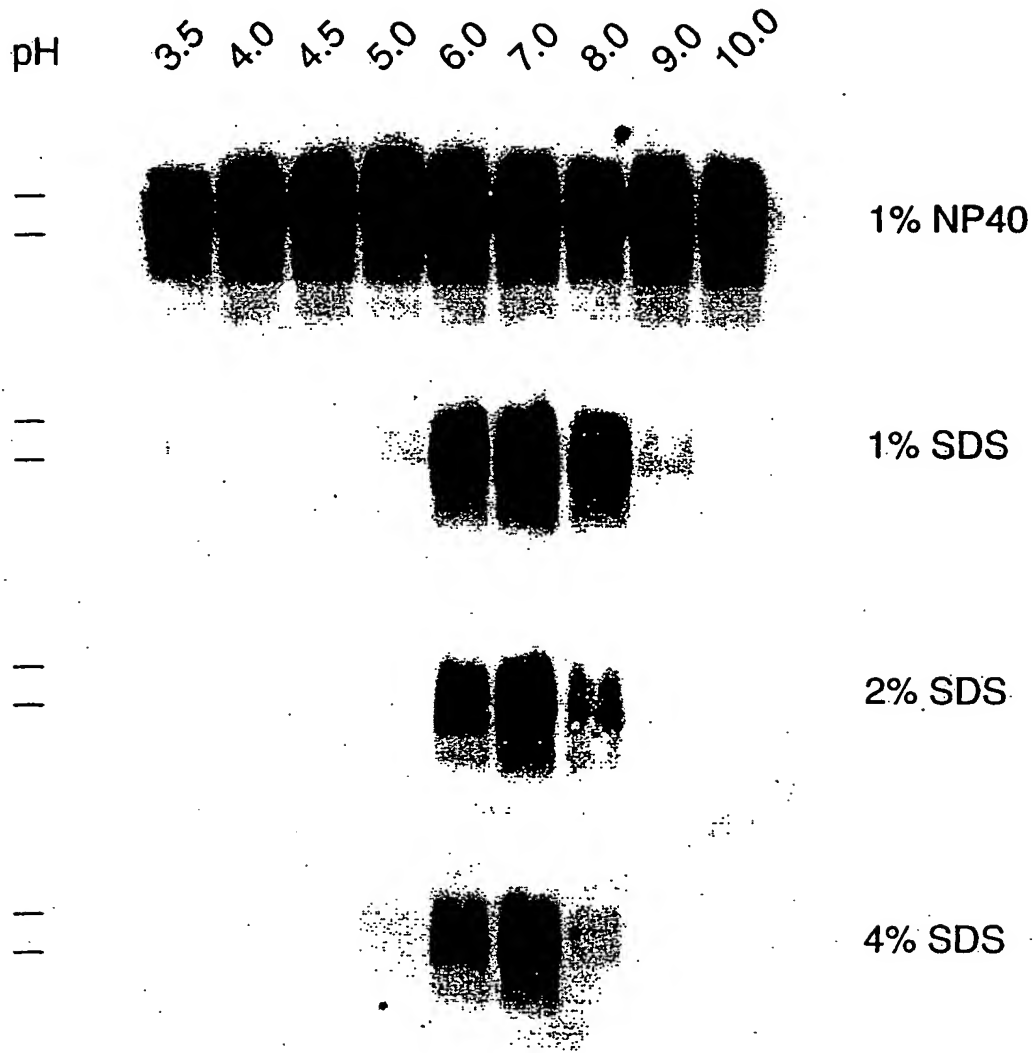| 1	2	3	4
− +	− +	− +	− +
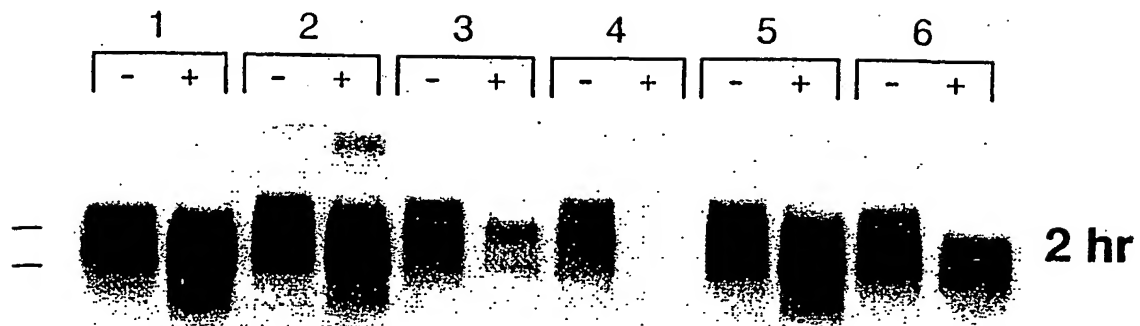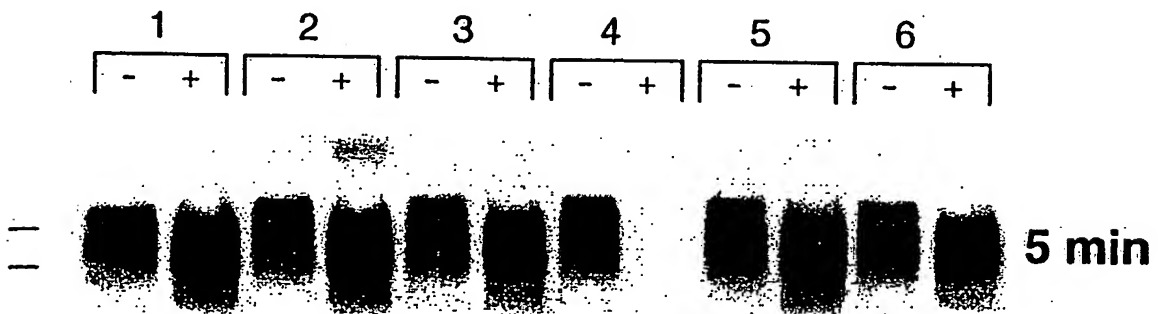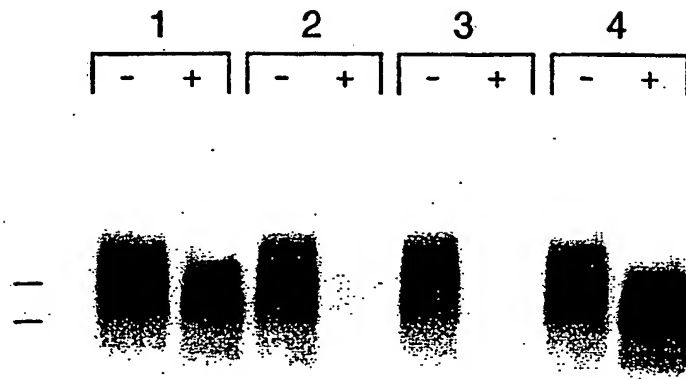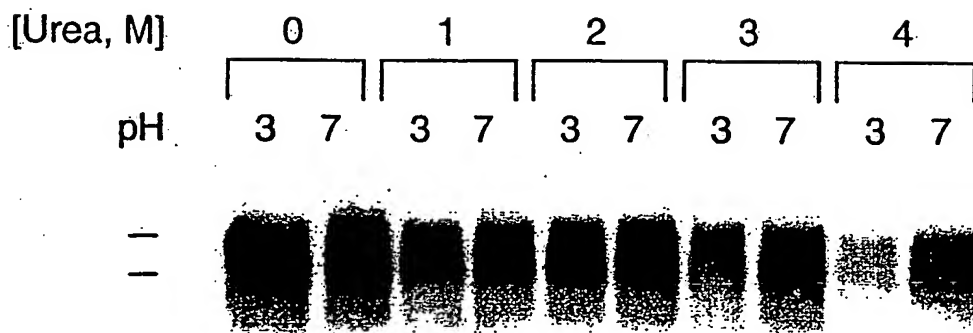
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